Wnt Signaling in Commissural Axon Guidance

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Table of Contents

1. Summary/Zusammenfassung

Summary

The population of dorsal commissural interneurons is a favoured model system to study the molecular mechanisms of axon guidance. The initial dorsal-to-ventral trajectory of commissural axons is well understood. However, the navigation of commissural axons after crossing the midline, when they abruptly turn into the longitudinal axis and extend towards the brain, is still poorly understood. Two distinct model organisms revealed two different molecular cues that guide these axons in the longitudinal axis. Wnt family members attract mouse commissural axon rostrally, while Sonic hedgehog (Shh) pushes chicken commissural axons towards the brain.

We could show that the role of Wnt proteins is conserved in the chick. The molecular mechanism of Wnt action, however, shows peculiarities not seen in the mouse. Expression analysis of *Wnt* genes led to three candidates, *Wnt4*, *Wnt5a*, and *Wnt7a*, which were further characterized. Loss of function and gain of function experiments revealed that Wnt5a and Wnt7a, but not Wnt4, are commissural axon guidance cues. This is in contrast to mouse, where Wnt4 is the major guidance force. But more importantly, *Wnt* transcripts were distributed evenly in the chick, while displaying gradients in the mouse. Secreted frizzled-related proteins are known Wnt antagonists. We could show that *Sfrp1* is expressed in a gradient and is able to modulate Wnt activity. Downregulation and upregulation of Sfrp1 phenocopies guidance errors seen after modulation of Wnt expression. Therefore, a Wnt activity gradient, shaped by Sfrp, guides postcrossing commissural axons in the chicken spinal cord.

Zusammenfassung

Die Zellpopulation der dorsalen Kommissuralneurone ist ein beliebtes Modellsystem, um die molekularen Mechanismen der axonalen Wegleitung zu studieren. Das initiale, dorso-ventral gerichtete Wachstum der Kommissuralaxone ist relative gut untersucht. Nachdem die Axone die embryonale Mittellinie überquert haben, wachsen sie, nach einem abrupten Richtungswechsel, in Richtung Gehirn. Über diesen Prozess ist noch wenig bekannt. Zwei verschiedene Modellorganismen haben zwei unterschiedliche molekulare Mechanismen für die Wegleitung in der Längsachse zu Tage gefördert. In der Maus werden Kommissuralaxone von Proteinen der Wnt Familie kopfwärts gezogen, während im Huhn dieselben Axone von Sonic hedgehog (Shh) Richtung Kopf gestossen werden.

Wir konnten zeigen, dass die Rolle der Wnt Proteine im Huhn und in der Maus vergleichbar sind. Die molekularen Mechanismen sind jedoch unterschiedlich. Die Analyse der Expressionsmuster verschiedener *Wnt* Gene führte zu drei Kandidaten, *Wnt4*, *Wnt5a* und *Wnt7a*, welche weiter charakterisiert wurden. Funktionelle Experimente demonstrierten, dass Wnt5a und Wnt7a, aber nicht Wnt4, eine Rolle in der axonalen Wegleitung haben. Dies steht im Gegensatz zur Maus, in welcher Wnt4 diese Funktion übernimmt. Noch schwerwiegender ist jedoch, dass *Wnt* Transkripte im Huhn gleichmässig verteilt waren, während in der Maus Transkriptionsgradienten gefunden wurden. Wir konnten zeigen, dass der Wnt Antagonist Sfrp1 in einem Gradienten exprimiert ist und die Wnt Aktivität regulieren kann. Modulation der Sfrp1 Expression führte zu ähnlichen Phänotypen wie wir sie bei den Wnt Proteinen gesehen haben. Wir schliessen daraus, dass im Rückenmark des Huhnes ein Wnt Aktivitätsgradient, geformt durch die Wirkung von Sfrp, die Wegfindung der Kommissuralaxone steuert.

2. Introduction

Axon guidance: historical considerations

The central nervous system is a highly complex organ. Whether it is composed of just 302 neurons in the case of Caenorhabditis elegans or whether several hundred billions of nerve cells need to be wired as in the case of the human brain, the problems to be solved during development are basically always the same. The research field that tries to understand the mechanisms of brain wiring is called axon guidance. Axons are processes of nerve cells, which in the case of projection neurons extend over long distances, to connect to the appropriate target cells. In contrast to the fine tuning of synaptic contacts, axon guidance is a process which is largely activity-independent (Goodman and Shatz, 1993). The growth cone, the leading edge of a growing axon, is the structure that senses the environment and guides axons through developing tissues. It was noticed for the first time over one hundred years ago, by Santiago Ramon y Cajal (1852-1934), a Spanish neuroanatomist and physician. By analyzing histological sections of nerve tissue he found that axons are equipped with a specialized polymorphic structure at their distal ends. He subsequently named them "cono de creminiento" (growth cone). Ramon y Cajal even went further and hypothesized that growth cones were the structures that sense chemical cues from the environment (chemotactism or long-range guidance) and navigate axons to their appropriate target (Ramon y Cajal, 1892). At the time, his "neurotropic hypothesis", however, could not be addressed experimentally because of technical limitations. Later, the introduction of in vitro experiments showed that axons need a solid substrate for their growth (Weiss, 1934). This finding pushed the idea of "contact guidance" and led to the hypothesis that axons are guided by repulsive and attractive substrates and therefore navigate according to preformed "itineraries" (blueprint hypothesis: (Singer et al., 1979)). One by one, molecular cues, short-range guidance molecules, were identified. They belonged to cell adhesion molecules of the Ig superfamily (IgCAMs), the Cadherin family,

the Integrin family, extracellular matrix molecules (ECM) and Proteoglycans (PG) (Bovolenta, 2005). Subsequently in vitro assays were established that revived Ramon y Cajals idea of chemotropism and showed that neurites are specifically attracted by their target or intermediate target tissue (Lumsden and Davies, 1983; Tessier-Lavigne et al., 1988). The identity of the guidance cues remained elusive however. Then, in the 1990s the search for guidance molecules culminated in the identification of several families of conserved axon guidance cues: the Netrins, Slits, Semaphorins, and Ephrins (Figure 1.3). Last but not least, the set of well characterized guidance molecules was complemented by the surprising discovery that morphogens also act as molecular cues for growing axons.

Guidance mechanisms

The complex task of leading axons through developing tissue is governed by four molecular mechanisms and facilitated by intermediate targets or guidepost cells and fasciculation/defasciculation with preformed axonal tracts (Tessier-Lavigne and Goodman, 1996). Diffusible axon guidance cues can mediate their action over long distances and be either attractive or repulsive (Figure 1.1). These mechanisms are called long-range attraction and repulsion or chemoattraction and chemorepulsion. On the other hand, molecular cues which are acting locally can either create an adhesive or repulsive substrate (Figure 1.1). These mechanisms are called short-range adhesion and repulsion or contact adhesion and contact repulsion. In the first case, long-range guidance molecules are present in concentration gradients and axons grow towards the source, the highest concentration, of a chemical cue or are repelled away from it. In vitro experiments suggested that a gradient as shallow as 0.1% across the growth cone diameter can be detected (Rosoff et al., 2004). In the second case, short-range guidance molecules provide an adhesive substrate that builds a highway for extending axons or a repulsive substrate that provides borders along permissive tissue. Contact repulsion might, at first thought, sound paradox, since

it is initiated, of course, by often strong ligand receptor binding, or adhesion. It was suggested however, that repulsion is enabled by metalloprotease-mediated protein clipping (Hattori et al., 2000) or by ligand receptor internalization (Zimmer et al., 2003). Most importantly, these four mechanisms are not mutually exclusive, but act in concert.

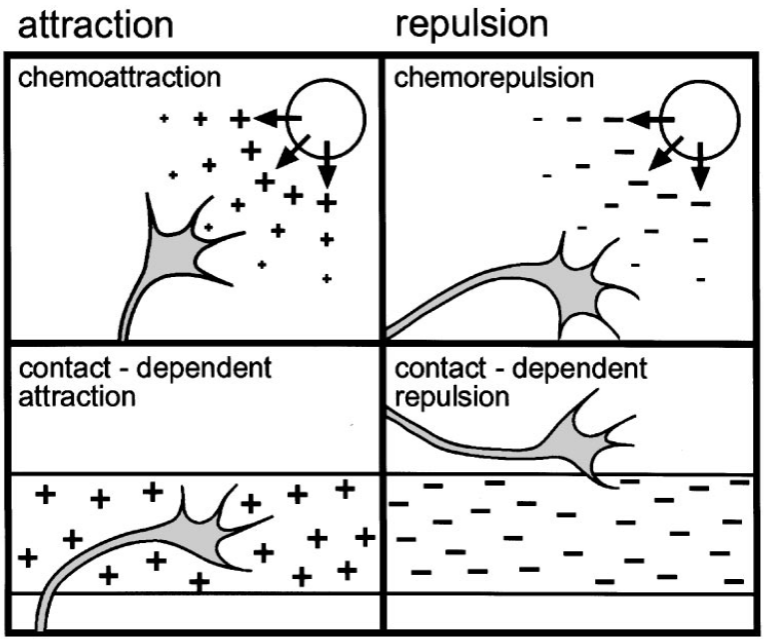

Figure 1.1 The four guidance mechanisms. Long-range cues mediate chemoattraction or chemorepulsion. They are locally secreted and attract or repel growing axons towards or away from their source. Short-range cues mediate contact-dependent attraction or repulsion. They form tracts of adhesive or borders of repulsive substrate. (From Muller, 1999.)

The axonal trajectory is segmented into short sequences of pathfinding. Before reaching the final target axons encounter intermediate targets or choice points. These intermediate targets can be either single cells, often referred to as guidepost cells (Bentley and Caudy, 1983; Caudy and Bentley, 1986) or more complexly structured tissues (as the midline in vertebrates and invertebrates; e.g. (Kaprielian et al., 2001)). Intermediate targets not only help to cut the axonal trajectory into short segments but also have an impact on the sensitivity of growth cones. For instance, it is known that the expression and the localization of proteins in and on commissural neurons changes at the timepoint of midline contact (e.g.: Hhip: (Bourikas et al., 2005); Robos in rodents: (Chen et al.,

2008b); PI3K: (Wolf et al., 2008); Comm in Drosophila: (Yang et al., 2009); Robo1/RabGDI in Philipp et al., submitted). However, the molecular mechanisms that are responsible for these switches remain poorly defined (Avraham et al., 2009; Wilson et al., 2008).

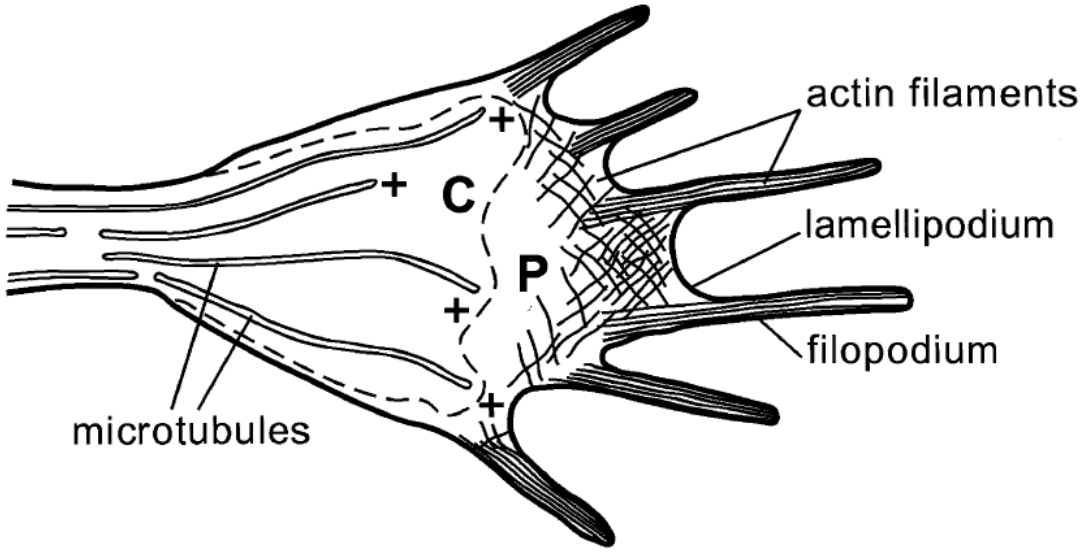

Figure 1.2 The growth cone. The organelle-rich central domain (C) is packed with microtubules. The peripheral domain (P) is dominated by actin filaments organized in tight bundles (filopodium) or in a dense meshwork of filaments (lamellipodium). (From Mueller, 1999.)

While pioneer axons need to find their way in an axon-free environment, later projecting axons encounter a scaffold of nerve fibers (fascicles) along their path. By fasciculating with these preexisting highways, and switching between different highways through defasciculation and fasciculation, axons find their way in the growing embryo (labeled-pathway hypothesis, (Raper et al., 1983)).

As correctly suggested by Ramon y Cajal more than 100 years ago, the intricate task of sensing the environmental cues is mediated by the tip of the axon, the growth cone (Figure 1.2). Its hand like structure reflects its function during development. The proximal part of the "palm" is called central domain or C-Zone. It mainly contains microtubules whose plus ends point towards the tip of the "hand". The distal part is the peripheral domain or P-Zone. The P-Zone can

appear as a rather flat lamellipodial or finger-like filopodial structure. The lamellipodium contains of a meshwork of actin-filaments and a few microtubules from the C-Zone reaching into the P-Zone. On the other hand, the filopodia are formed by actin filaments organized in actin-fibers. Filopodia can become very long, reaching into the environment sensing the surrounding. The actin cytoskeleton is in a constant retrograde flow because of its myosin-mediated interaction with microtubules. At the same time actin filaments polymerize distally and depolymerize proximally. The adhesion to a substrate and its coupling to the cytoskeleton cause an attenuation of the F-actin flow. The subsequent tension that occurs because of the F-actin pulling on microtubules and the distal polymerization of actin leads to growth cone protrusion. Axon guidance cues influence the behavior of neurites by providing adhesion (or preventing it) and by changing the kinetics of retrograde F-actin flow and/or actin and microtubule polymerization/depolymerization.

Classical axon guidance molecules

Netrins

A small family of axon guidance cues, related to the ECM molecule Laminin, is made up by the Netrins. Netrins act as bifunctional long-range cues and where identified during the search for an attractant of vertebrate commissural axons. While attractive to some axonal populations (e.g. commissural axons, (Kennedy et al., 1994)), they mediate repulsion to others (e.g. trochlear motor axons, (Colamarino and Tessier-Lavigne, 1995)). Four family members are known in rodents and humans (Netrin-1, -3, -4, and -G1; (Manitt and Kennedy, 2002)), two in Drosophila (Netrin-A, and –B, (Harris et al., 1996)) and one in C.elegans (UNC6, (Hedgecock et al., 1990)). Netrins transmit a guidance signal by means of their receptors DCC and UNC5. The effect of Netrin is receptor-context dependent: DCC transduces mainly attraction, while UNC5 alone or in concert with DCC mediates repulsion. Two DCC receptors are known in vertebrates,

DCC and Neogenin, one in Drosophila and one in C.elegans, Frazzled and UNC40, respectively. UNC5 is represented by just one gene in invertebrates, while vertebrates have three, UNC5H1-3 (Yu and Bargmann, 2001).
Despite the high affinity of Netrin to cell membranes (Kennedy et al., 1994; Serafini et al., 1996), a diffusion gradient in rodents and chick could be visualized recently (Kennedy et al., 2006). How and whether Netrin diffusion is regulated is still unknown. Moreover, Netrin-1 was shown to locally regulate protein levels in growth cones by activating protein synthesis and triggering protein degradation, a function which was important for the induction of chemotropic responses (Campbell and Holt, 2001). The outgrowth-promoting effect of Netrin, on the other hand, was shown to be Calcineurin/NF-AT-dependent (Graef et al., 2003).

Slits

The Slit-Robo system was identified in Drosophila by a genetic screen for midline guidance defects (Kidd et al., 1999; Kidd et al., 1998; Seeger et al., 1993). The Roundabout (Robo) phenotype, crossing and re-crossing of ipsilateral and commissural axons, suggested the presence of a repellent cue at the midline (Seeger et al., 1993). At the same time, another mutation, Commissureless (Comm), suggested the presence of an attractive signaling system complementing repulsive Roundabout (Seeger et al., 1993). Commissureless, however, turned out to regulate the surface expression of Robo. Removal of Comm causes Robo surface localization and constitutive precrossing repulsion, explaining why there are virtually no commissures in Comm mutants (Gilestro, 2008; Keleman et al., 2002). Subsequently, midline-derived Slit was identified as the ligand mediating repulsion through Robo receptors (Kidd et al., 1999). Comm expression, in turn, seems to be regulated by the Netrin receptor Frazzled/DCC in a Netrin-independent fashion (Yang et al., 2009). The Drosophila genome encodes for three Robo receptors (Robo1-3) which mediate the lateral positioning of axons in the longitudinal axis (Rajagopalan et al., 2000; Simpson et al., 2000). Slit-Robo function at the midline is conserved in

vertebrates (Brose et al., 1999; Long et al., 2004). Interestingly, vertebrate genomes do not have a Comm orthologue.

Another well-characterized function of Slit-Robo is the guidance of retinal ganglion cell axons at the optic chiasm where they form repulsive borders for ipsi- and contralaterally projecting axons (Rasband et al., 2003). The distinction of ipsi- and contralateral projections is provided by another conserved receptor-ligand pair of axon guidance cues, the Ephrins and their cognate Eph receptors (Rasband et al., 2003; Williams et al., 2003).

Ephrins

In 1963, Sperry postulated that gradients of molecules in retina and tectum (and not unique labels for an axon and its target cell) regulate topographic mapping of retinal ganglion cell axons in the tectum (Chemoaffinity hypothesis: (Sperry, 1963)). More than 30 years later, Ephrins and Eph receptors (receptor tyrosine kinases) were identified as the graded factors providing positional information for retino-tectal mapping (Cheng et al., 1995; Drescher et al., 1995). Ephrins and Eph receptors come in two flavors. The GPI-anchored Ephrin-As bind EphA receptors, while transmembrane Ephrin-Bs bind EphB receptors. Ephrins are grouped based on their membrane attachment. Classification of the Eph family of receptor tyrosine kinases (RTKs) is based on binding-specificities to Ephrins which correlate with sequence similarities between Eph receptors. EphA4 is ‘the exception that proves the rule’ since it can also bind to Ephrin-Bs (Gale et al., 1996; Wilkinson, 2001). Vertebrate genomes encode 14 or more Ephs and 8 Ephrins (3 Ephrins-As and 5 Ephrin-Bs). A single Ephrin and a single Eph receptor were identified in Drosophila, while four Ephrins and one Eph were found in the C.elegans genome (Scully et al., 1999; Wang et al., 1999; Wilkinson, 2001). Intriguingly, bi-directional signaling was reported for both classes of Ephrin/Eph ligand-receptor pairs (Grunwald and Klein, 2002). Ephrins and Ephs can be either attractive or repulsive (Dickson, 2002).

Semaphorins

The first two Semaphorins were identified as guidance cues for grasshopper Ti1 axons (Sema-1a, initially called Fasciclin IV: (Kolodkin et al., 1992)) and as potent inducers of growth cone paralysis or collapse (therefore called Collapsin, now known as Sema3a: (Luo et al., 1993)). Semaphorins are divided into 8 classes depending on structure and species. Class 1 (transmembrane) and class 2 (secreted) Semaphorins are invertebrate specific while class 3 (secreted), classes 4-6 (transmembrane), and class 7 (GPI-anchored) Semaphorins are encoded by vertebrate genomes. The eighth class, class V, is virally encoded. All Semaphorins are characterized by the N-terminal 500 amino acids that form the Sema domain (Committee, 1999; Gherardi et al., 2004). Secreted Semaphorins signal by means of a receptor complex including Plexins and Neuropilins. Membrane-bound Semaphorins signal through Plexin receptors alone (Tamagnone and Comoglio, 2000; Zhou et al., 2008). Neuropilins do not contain signaling motifs within their short cytoplasmic tail suggesting that they can not signal on their own (Dickson, 2002; Raper, 2000). The receptor complex by which Semaphorins signal was shown to contain many more components than just Plexins and Neuropilins (Zhou et al., 2008). For instance, it was shown that Sema3a repels cortical axons in an L1-dependent manner (Castellani et al., 2000) and Sema4D can signal by means of the receptor tyrosine kinase MET (known as a hepatocyte growth factor/scatter factor (HGF/SF) receptor; (Giordano et al., 2002)). In addition, the receptor tyrosine kinase OTK (off-track) was identified in Drosophila to associate with Plexins and mediate axon guidance downstream of Sema1a (Winberg et al., 2001). Similar to Ephrins, bidirectional signaling was shown for class 4 and class 6 Semaphorins (Delaire et al., 1998; Godenschwege et al., 2002; Tamagnone and Comoglio, 2000; Toyofuku et al., 2004; Zhou et al., 2008).

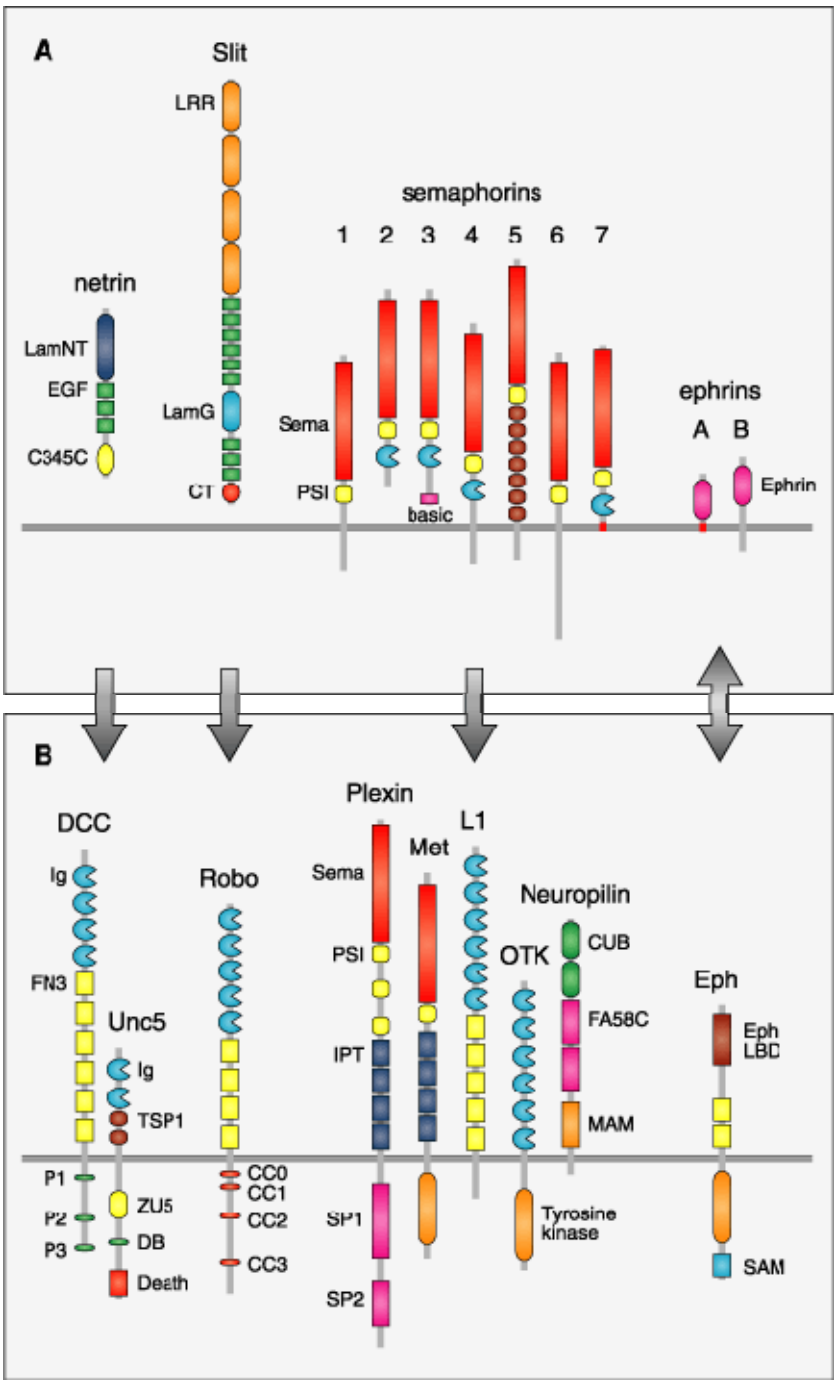

Figure 1.3 Classical axon guidance cues (A) and their receptors (B). Domain names are from SMART (http://smart.embl-heidelberg.de). P1 to P3, DB (DCC-binding), CC0 to CC3, and SP1 and SP2 indicate conserved regions in the cytoplasmic domains of DCC, UNC-5, Robo, and Plexin receptors, respectively. (From Dickson, 2002.)

Signaling downstream of axon guidance receptors

Signal transduction initiated by ligand-receptor interactions resulting in axonal attraction or repulsion converges intracellularly to the Rho family of small GTPases (Figure 1.4; (Dickson, 2002; Mueller, 1999)). Rho family GTPases, a subfamily of the Ras superfamily, consists of over a dozen members in mammals. The three best-studied members, however, are RhoA (Ras homologous member A), Rac1 (Ras-related C3 botulinum toxin substrate 1), and Cdc42 (cell division cycle 42) (Luo, 2000). Rho GTPases were first identified in the regulation of fibroblast cytoskeleton dynamics (Hall, 1998). Swiss 3T3 fibroblasts reacted differently upon the activation of distinct Rho GTPases. RhoA activation caused assembly of stress fibers (contractile actin-myosin fibers) and focal adhesion complexes. Rac1 and Cdc42 induced the formation of lamellipodia and filopodia, respectively. In neurons, activation of Rac1 and Cdc42 has a similar effect on neurites. RhoA activation, in contrast, causes neurite retraction (Hall, 1998). Small GTPases function as molecular switches: active while bound to GTP, the intrinsic GTPase activity hydrolyses GTP to GDP, leading to the inactive GDP-bound state. Downstream they regulate actin dynamics via activation of kinase cascades culminating in the control of, or by direct control of actin polymerization/depolymerization factors (Mueller, 1999). The activity of Rho GTPases is adjusted by GTPase-activating proteins (GAP; GAPs facilitate GTP hydrolysis), Guanine nucleotide exchange factors (GEF; GEFs activate GTPases by GDP/GTP exchange), and Guanine nucleotide dissociation inhibitors (GDI; GDIs maintain the inactive GDP-bound state). Axon guidance receptors regulate cytoskeleton dynamics by acting on several levels within transduction pathways. While some PlexinBs directly bind Rac1 in a GTP-dependent manner, Robo receptors can signal by means of dedicated GAPs, the Slit-Robo GAPs (srGAPs). Eph receptors on the other hand are able to interact with GAPs and GEFs (RasGAP, Ephexin) and Src family kinases (Grunwald and Klein, 2002). Tyrosine phosphorylation by Src family kinases allows recruitment of adaptor proteins and subsequent activation of cytoskeleton regulators, e.g.

FAK (focal adhesion kinase). Morphogens can signal via Rho GTPases and the activation of Src family kinases (Schlessinger et al., 2009; Wouda et al., 2008; Yam et al., 2009).

Using dissociated Xenopus spinal neurons it was shown that Calcium signaling is crucial for Netrin-1-mediated growth cone turning (Hong et al., 2000). Furthermore, earlier work implicated Calcium signaling in axon extension (i.e. (Kater et al., 1988)). Interestingly, as demonstrated by Hong and colleagues, growth cone turning can be induced in the absence of Netrin-1 by applying a ryanodine gradient. Ryanodine inhibits calcium channels, called ryanodine receptors, located in the endoplasmatic reticulum. Moreover, the axons were repelled (high) or attracted (low) depending on the concentration of ryanodine added to create the microgradient. It is likely that Calcium regulates cytoskeleton dynamics by the activation of Ca^{2+}-dependent kinases, e.g. CaMKII (Ca^{2+}/Calmodulin-dependent kinase II) and PKC (Protein Kinase C), phosphatases (e.g. Calcineurin), and other proteins, e.g. adenylyl cyclases (Henley and Poo, 2004; Zheng and Poo, 2007).

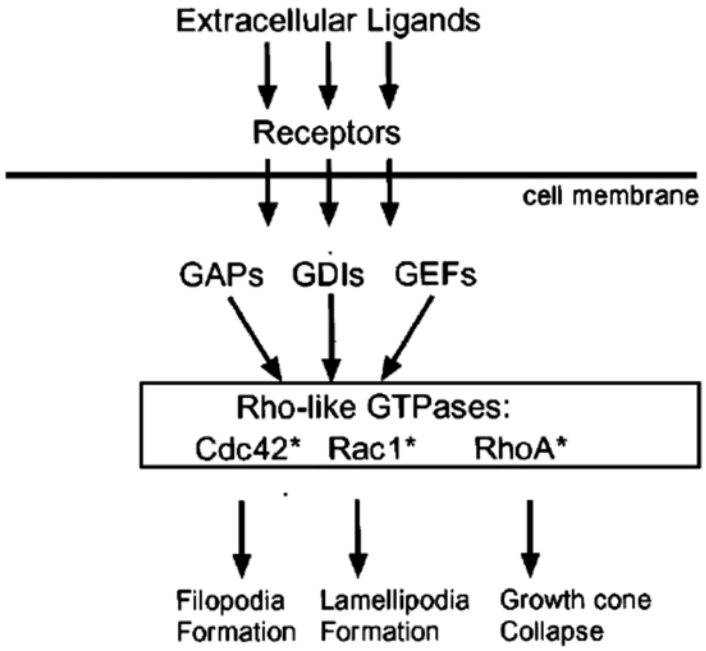

Figure 1.4 Intracellular signaling cascades. Activation of axon guidance receptors converges intracellularly in the activation of Rho GTPases. Rho GTPases have different impacts on the structure of the growth cones. While Cdc42 and Rac1 induce filopodia and lamellipodia, respectively, RhoA induces growth cone collapse. (Adapted from Mueller, 1999.)

The ability of cyclic nucleotides to modulate guidance responses was first shown in Xenopus spinal neurons (Song et al., 1998). Collapsin-1/Semaphorin-3 induced growth cone collapse was converted to attraction after cGMP pathway activation. Activation of cAMP pathway could modulate the cGMP-dependent conversion of the turning response. Subsequent studies refined the emerging picture: relative concentrations of cAMP and cGMP regulate Ca^{2+} channels via PKA and PKG, respectively (Nishiyama et al., 2003). Calcium then regulates cytoskeleton dynamic as described above. An intriguing example of the modulating activity of cyclic nucleotides was given by the study of pyramidal cells from the cortex (Polleux et al., 2000). Semaphorin3a acts as a chemoattractant for growing dendrites of certain neurons and simultaneously repels the axons of the very same neurons. In vitro experiments suggest that this differential response is due to asymmetric localization of soluble Guanylate cyclase, the enzyme producing cGMP. In general, higher cAMP and lower cGMP levels favor attraction while lower cAMP and higher cGMP is associated with repulsion (Dickson, 2002).

Morphogens

Morphogens are proteins which provide positional information. Produced and secreted from a particular source they create concentration gradients by diffusion. Diffusion of morphogens is not simply passive but often actively regulated by extracellular transport mechanisms. By binding to receptor proteins on target cells, intracellular signal transduction pathways are elicited, which, depending on the concentration of the morphogen, regulate gene transcription. The initially relatively fuzzy induction of gene expression pattern is refined by cross-interactions of the regulated genes. Strictly speaking, these criteria are only met by members of the Hedgehog, Wnt, and Bone morphogenetic protein (BMP) families. A more loosely formulated definition would be that morphogens must directly regulate gene transcription at a distance, and do so in a

concentration-dependent fashion (Vincent and Briscoe, 2001). These two criteria would include the Drosophila transcription factors Bicoid and Hunchback, the first morphogens to be identified.
All known morphogens, Hedgehogs, Wnts, and BMPs turned out to be axon guidance molecules and at least the guidance function of Wnts is conserved during evolution.

BMPs

Members of the TGFβ superfamily, BMPs, were shown to be expressed in the roof plate and to be important for the patterning of the dorsal spinal cord (Lee and Jessell, 1999). Soon after their morphogenetic action they are involved in guiding commissural axons towards the floor plate by providing a repulsive cue (Augsburger et al., 1999). Knockout mice and in vitro studies suggested that Bmp7 and Gdf7 (growth differentiation factor) work as heterodimers to push commissural axons ventrally (Butler and Dodd, 2003). In cell specification processes, BMPs signal through a receptor complex of type I and type II receptor serine/threonine kinases. After the type II receptor-mediated phosphorylation of the type I receptor the signal is relayed into the nucleus by the Smad family of proteins (Attisano and Wrana, 2002). BMP receptors of the type I (BMPRI) are also used during axon guidance. However, only BMPRIB mediates commissural axon repulsion while cell specification is coordinated by the conjoint activation of BMPRIA and BMPRIB (Yamauchi et al., 2008). This suggests that differential cellular output, that is cell specification versus axon guidance, is controlled by the activation of distinct sets of receptors. The intracellular pathway regulating the axonal response upon BMP ligand binding remains by and large elusive. It was shown, however, that type II BMP receptors regulate actin cytoskeleton via the activation of LIM kinases (Sanchez-Camacho and Bovolenta, 2009).

Shh

Members of the Hedgehog (Hh) family are well known for their function in pattern formation. A classic example is the specification of cellular identities in the vertebrate spinal cord (Dessaud et al., 2008). In vertebrates, three genes that belong to the *Hedgehog* family have been described: *Indian hedgehog, Desert hedgehog, and Sonic hedgehog*. The Drosophila genome encodes for one *Hh* gene. The nematode Caenorhabditis elegans has no *Hh* orthologue, probably due to a loss of the gene (Burglin, 2008).

Sonic hedgehog (Shh), one of three vertebrate Hh, was identified as an axon guidance cue by Charron and colleagues in 2003 (Charron et al., 2003). The morphogenetic gradient of Shh is 'recycled' to attract commissural axons towards the ventral midline. Shh attracts commissural axons without changing the overall outgrowth (Charron et al., 2003; Yam et al., 2009). The chemoattraction is mediated by the canonical Hh receptor Smoothened. Intriguingly, there is no evidence for the involvement of Patched in this process. However, another Hh receptor, Boc, was shown to be essential for Shh to attract commissural axons (Okada et al., 2006). Moreover, preliminary observations suggest that Gas1, a positive regulator of Shh signaling, is involved in precrossing commissural axon guidance (Allen et al., 2007). Only shortly after Shh mediates chemoattraction towards the ventral midline, a local anteroposterior gradient of Shh in the floor plate guides commissural axons rostrally into the longitudinal axis (Bourikas et al., 2005). Interestingly, this time Shh acts as repulsive cue via Hhip (Hedgehog-interacting protein) in a Ptch/Smo-independent mechanism. It seems therefore that the effect of Shh on axonal behavior depends on differential expression of Hh receptors. Moreover, retinal ganglion cell (RGC) axons display a similar behavior, depending on the population of RGCs (Sanchez-Camacho and Bovolenta, 2008). Shh is outgrowth promoting for ipsilaterally projecting RGC axons while inhibiting the growth of contralaterally projecting RGCs. Again, this behavioral discrepancy possibly occurs due to differential expression of the Hh

receptors Ptch2 and Boc. Recent evidence suggests that, at least in commissural axons, Shh regulates the cytoskeleton through Src family kinases (Yam et al., 2009).

Wnt: Wnt signaling and axon guidance

Introduction

Developmental processes are governed by only a handful of signaling pathways. The Wnt signaling pathway is probably the best studied among these molecular cascades. It nicely exemplifies how one single molecular transduction pathway can regulate many different cellular functions (e.g. differentiation, cell migration), how these functions are evolutionary conserved among phyla (e.g. axon guidance in invertebrate and vertebrates), and how signaling mechanisms are recycled during ontogeny (e.g. differentiation, axon guidance).

More than 20 years ago int-1, a gene locus that caused mammary carcinomas after genomic integration of a mouse mammary tumor virus (MMTV) and wingless, a drosophila segment polarity gene, were shown to be orthologues (Baker, 1987; Nusse and Varmus, 1982; Rijsewijk et al., 1987). Subsequently, the Wnt gene family was named after the two initial founders wingless and int-1 (Nusse et al., 1991). It comprises 19 members in vertebrates (www.stanford.edu/~rnusse/wntwindow.html). In the years thereafter, a complex Wnt signaling network was unraveled. Today a plethora of research papers and reviews deal with Wnt signaling ranging from proliferation and differentiation in early embryonic development to human degenerative diseases (Clevers, 2006; Logan and Nusse, 2004; MacDonald et al., 2009).

Wnts are secreted glycoproteins that can induce intracellular signal transduction pathways leading to several distinct outcomes. For a long time little was known about the structure of Wnts due to their poor solubility. All family members share 23 to 24 conserved cysteine residues suggesting the occurrence of disulfide

bonds (Miller, 2002). Moreover, purification of murine Wnt3a revealed two lipid modifications which are important for function and secretion (Takada et al., 2006; Willert et al., 2003). These two lipidations probably explain the high hydrophobicity of Wnt proteins (Hausmann et al., 2007). In line with these findings, Wnt proteins tend to associate with the plasma membrane and glycosaminoglycans in the ECM (Bradley and Brown, 1990; Reichsman et al., 1996). It was suggested that extracellular movement of Wnt proteins is facilitated by binding to lipoprotein particles (Panakova et al., 2005), the formation of multimers (Katanaev et al., 2008), or proteoglycans (Lin, 2004).

β-catenin-dependent Wnt signaling

Wnt signaling is segregated into three pathways: Wnt/β-catenin, Wnt/Calcium and Wnt/PCP signaling (Figure 1.5). The Wnt/β-catenin pathway is also called canonical signaling, while the Wnt/calcium and Wnt/PCP pathways are sometimes conjointly referred to as noncanonical signaling. However, more data is accumulating that these branches are components of a signaling network rather than separate cascades (Kestler and Kuhl, 2008).

Wnt/β-catenin signaling, the best characterized Wnt signaling pathway, regulates gene transcription. Signal transduction is initiated by Wnt binding to its cognate Frizzled receptor (Bhanot et al., 1996) and co-receptor Lrp5 or Lrp6 (low density lipoprotein receptor-related protein), called Arrow in Drosophila (Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000). Frizzled receptors, in vertebrates encoded by 10 genes, are seven-pass transmembrane receptors related to G-protein coupled receptors (GPCR) and contain an extracelluar N-terminal cysteine-rich domain (CRD) (Huang and Klein, 2004). In the absence of a Wnt ligand, a ‘destruction complex’ consisting of Axin, adenomatous polyposis coli (APC), β-catenin, casein kinase 1 (CK1), and glycogen synthase kinase 3 (GSK3) targets β-catenin via CK1 and GSK3-dependent phosphorylation for ubiquitination and proteasomal degradation. In the presence of Wnt, the scaffolding protein Axin is recruited to the ternary Wnt/Frz/LRP6 complex by the

action of Dishevelled (Dvl). Oligomerization of Dvl promotes the formation of Frz/LRP6 receptor complex clusters, so called signalosomes, enhanced Axin recruitment and finally a disruption of the destruction complex. Subsequently β-catenin accumulates, translocates to the nucleus and activates target gene expression together with transcription factors of the TCF/LEF family (T-cell factor/Lymphoid-enhancer factor) (MacDonald et al., 2009).

Wnt/Calcium pathway

Another Wnt signaling pathway was identified in zebrafish. The transparent zebrafish embryos revealed that Calcium signaling was augmented after overexpression of Xwnt-5a, but not Xwnt-8 (Slusarski et al., 1997b), and that this effect was mediated through Frizzled, heterotrimeric G-proteins, and phosphatidylinositol (PI) signaling (Slusarski et al., 1997a). In PI signaling an activated protein lipase C (PLC) cleaves phosphatidylinostiol-4,5-bisphosphate (PIP2) into the second messengers inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 regulates IP3 receptors (IP3R) located in the endoplasmatic reticulum causing the release of Calcium (Kuhl, 2004). Subsequently, the group of Randall Moon showed that Wnt signaling can indeed resemble PI signaling. They demonstrated that the Calcium-sensitive enzymes protein kinase C (PKC) (Sheldahl et al., 1999) and Ca^{2+}/calmodulin-dependent kinase II (CAMKII) (Kuhl et al., 2000) are activated in this pathway, and that Dvl acts downstream of Frizzled (and probably G-proteins) to activate PKC and CAMKII (Sheldahl et al., 2003). Furthermore it was shown that another calcium sensitive protein, the phosphatase Calcineurin, is involved in Wnt signaling. Saneyoshi and colleagues found that Wnt5a can stimulate translocation of nuclear factor of activated T-cells (NF-AT) transcription factors to the nucleus in a Calcium/Calcineurin-dependent mechanism (Saneyoshi et al., 2002). Another side-branch of the Wnt/Calcium pathway was uncovered by using a chimeric receptor made of the extracellular and membrane spanning parts of the serpentine β_2-adrenergic receptor (β_2-AR) and the intracellular loops and the C-

term of rat Frizzled-2. The activity of this chimeric receptor could be modulated by β_2-AR agonists and antagonists, circumventing the low solubility of Wnt proteins (Ahumada et al., 2002). Ahumada and colleagues found that Calcium increase was accompanied by a decrease of cGMP mediated by a cGMP-specific phosphodiesterase (PDE).

Wnt/planar cell polarity pathway

Planar cell polarity (PCP) refers to the establishment of a second axis of polarity in an already apico-basally polarized epithelium. However, PCP is not restricted to epithelial tissues, but occurs also in mesenchymal cells (Simons and Mlodzik, 2008). Examples of epithelial polarity established by PCP are Drosophila eye imaginal discs, with meticulously orientated ommatidia, or the hexagonal cells of the Drosophila wing epithelium, with single actin-filled protrusions (wing hairs) at the distal vertex. In vertebrates processes related to PCP in Drosophila are found: for instance convergent extension movements, orientation of inner ear sensory hair cells, and neural tube closure (Wang and Nathans, 2007). Several Wnts (Wnt5a, Wnt7a, and Wnt11) are implicated in the vertebrate PCP pathway (Dabdoub et al., 2003; Heisenberg et al., 2000; Kilian et al., 2003). Intriguingly however, to date there is no evidence for a function of Wnt ligands in Drosophila PCP (Simons and Mlodzik, 2008). Wing cells mutant for five Drosophila Wnt genes (the two remaining Wnt genes were shown to be not expressed) display no polarity phenotype and the frizzled mutant can be rescued by a frizzled lacking the CRD. Thus there seems to be no need for a Wnt ligand in Drosphila PCP (Chen et al., 2008a). The core components of the PCP pathway are Frizzled, Flamingo (aka Starry night, aka Celsr), Dishevelled, Diego (aka Inversin), Prickle, and Van Gogh (aka Strabismus). The gene names reflect the phenotypes seen in Drosophila or mouse after mutation of one of the core components. The orientation of wing hair or hair follicles is not completely randomized but displays whorls across the tissue, reminiscent of paintings by Vincent Van Gogh (i.e. Starry Night). But what happens at the molecular level in

PCP signaling? The initial step in PCP pathway is the asymmetric distribution of the core proteins, a process involving intra- and intercellular communication (Goodrich, 2008). While Frizzled and Dishevelled localize at one side of a cell in a planar sheet, i.e. distal in the Drosopohila wing epithelium, Prickle and Van Gogh concentrate on the other side, thus proximal in the wing epithelium. Flamingo and Diego appear to be evenly distributed. Subsequently, initiated at the side where Frizzled receptors are present, a downstream signaling cascade regulates cytoskeletal rearrangements via Dishevelled, Rho family GTPases, cJun N-terminal kinase (JNK), and other kinases (Boutros et al., 1998; Schlessinger et al., 2009; Strutt et al., 1997).

Canonical or noncanonical? From very early on researchers tried to classify Wnt proteins according to their developmental and cellular activities. Experiments with Xenopus embryos revealed that int-1 (now Wnt1) had the ability to induce the formation of a secondary axis (McMahon and Moon, 1989). Using this axis duplication assay Wnt proteins were subdivided into two classes: those that did induce a second axis (Wnt1, Wnt3a, Wnt8) and those that did not (Wnt4, Wnt5a, Wnt11) (reviewed in (Kuhl, 2002)). Similar results were obtained with other assays, i.e. the transformation of C57MG cells and the stabilization of β-catenin (Shimizu et al., 1997; Wong et al., 1994). Initially, the classification according to these assays was consistent. Some Wnts were canonical, inducing a secondary axis in Xenopus, causing a stabilization of β-catenin and so on. Other Wnt proteins were noncanonical. The situation turned out to be more complicated, however. Wnt5a, a noncanonical Wnt normally unable to activate β-catenin pathway (Takada et al., 2005), can initiate canonical pathway if LRP5 and/or Frz4 are/is co-overexpressed in 293T cells (Mikels and Nusse, 2006). Furthermore, Wnt5a can induce axis duplication in Xenopus if coexpressed with Frz5 (He et al., 1997; Holmen et al., 2002).

Currently, it is not known whether Wnts bind selectively to certain Frz or whether they are promiscuous (Angers and Moon, 2009). However, several lines of evidence suggest that pathway specificity is conferred by the recruitment of

different coreceptors and cofactors. β-catenin-dependent Wnt/wingless signaling requires LRP/Arrow together with Frizzled (Schweizer and Varmus, 2003). A β-catenin-independent pathway is activated through Frizzled, the receptor tyrosine kinase (RTK) Ror2 (Hikasa et al., 2002; Oishi et al., 2003) and Cthrc1 (Yamamoto et al., 2008). Furthermore, it is well appreciated that canonical signaling is negatively regulated by noncanonical Wnt signaling (i.e. (Mikels and Nusse, 2006; Topol et al., 2003; Torres et al., 1996; Yamamoto et al., 2008). The mechanisms of inhibition are still poorly understood.

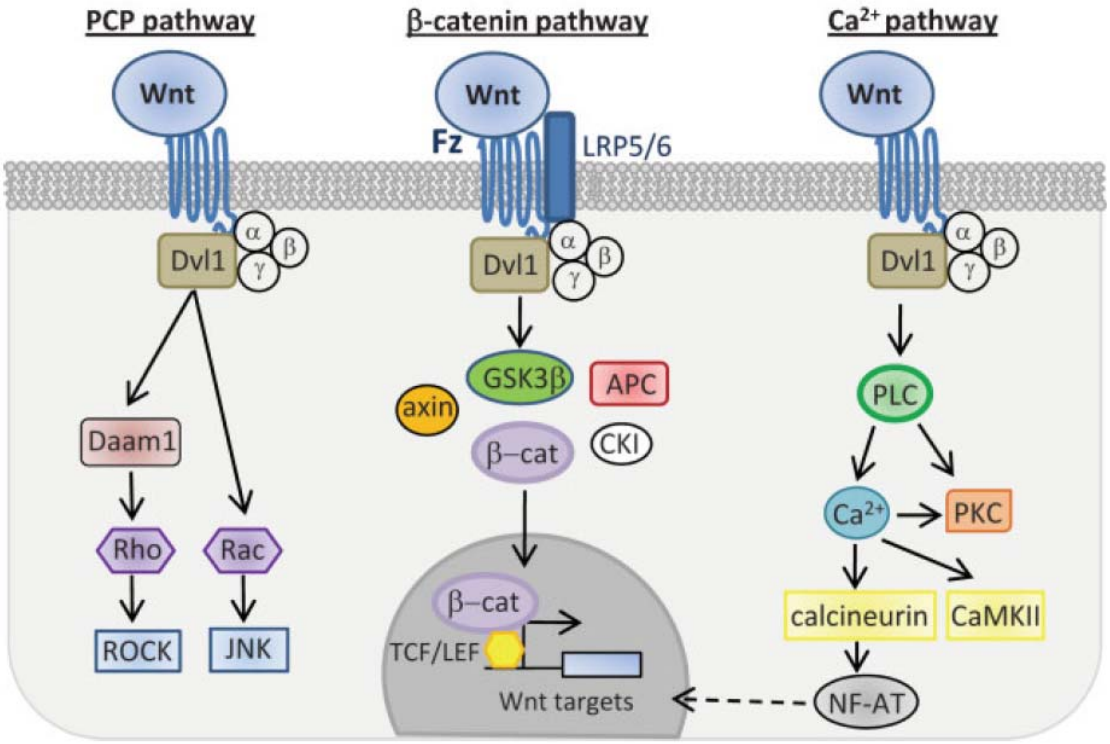

Figure 1.5 Canonical and non-canonical Wnt pathways (from Sanchez-Camacho and Bovolenta, 2009).

Wnt signaling and commissural axon guidance

The function of the nervous system crucially depends on its highly organized connectivity. To establish neural connections axons need external cues guiding developing neurites to their appropriate targets. In general, axon guidance cues

regulate growth cone behavior by modulating actin dynamics, retrograde actin flow (myosin-mediated interactions with microtubules), and microtubules stability (Dickson, 2002). The vertebrate population of dorsal commissural axons is a prominent model to study the molecular mechanisms of axon guidance. The initial dorso-ventral growth of commissural axons is mediated by several guidance cues and described fairly well. After crossing the midline, the axons make a sharp turn and steer rostrally, towards the brain. Until recently, nothing was known about the factors guiding commissural axon in the longitudinal axis. In 2003, Lyuksyutova and colleagues showed in a clever in vitro approach that a secreted guidance cue, rather than a membrane-tethered one, is responsible for the sharp anterior turn (Lyuksyutova et al., 2003). After screening several potential candidates they found the morphogen family of Wnt proteins as potent inducers of postcrossing commissural axon growth in mice. Wnt4 was not only expressed in a gradient at the right place and time but was indeed able to redirect axonal growth in collagen cultures. This effect is mediated by Frizzled3, since commissural axons of $Fzd3^{-/-}$ mice showed random turning after midline crossing. In 2005, Bourikas and colleagues demonstrated that in the chick, Sonic hedgehog (Shh), another morphogen, guided postcrossing commissural axons along the longitudinal axis (Bourikas et al., 2005). Shh was shown to be expressed in an antero-posterior gradient and accordingly acted as repulsive cue in vivo and in vitro. The question arose whether mouse and chick use different morphogens to guide commissural axons or whether Shh and Wnt cooperate in this process (Stoeckli, 2006). A follow up study in the chick indeed found that Wnt function was conserved in axon guidance (this thesis). Intriguingly, important differences were seen between mouse and chick. In the chick, Wnt5a was the most potent family member, in redirecting axonal growth after overexpression in vivo. In addition, Wnt4, the major player in mouse, was not expressed appropriately in the chick and downregulation did not induce axon guidance phenotypes. Most importantly, no longitudinal expression gradients were found for Wnt transcripts. It turned out that secreted frizzled-related proteins (Sfrp), known Wnt antagonists (Kawano and Kypta, 2002), are expressed in gradients

and are able to block Wnt-mediated axon attraction. These findings suggested that a Wnt activity gradient, rather than a transcriptional gradient, guides postcommissural axons in the chick. However, the intracellular pathway guiding postcommissural axon into the longitudinal axis is still poorly understood. A first hint was given by Wolf and colleagues, when they showed that in the mouse, postcrossing axons are guided by a mechanism involving atypical protein kinase C (aPKC, Figure 1.6A; (Wolf et al., 2008)). Atypical PKC has been linked to cell motility before and is known for its function in apical basal polarity (see below). Whether any known Wnt signaling cascade is involved in commissural axon guidance remains elusive.

Is Wnt signaling to the nucleus involved in axon guidance? Directing axons through the developing tissue requires fast local responses, suggesting that guidance mechanisms are independent of gene transcription. Indeed it was shown that growth cones of retinal ganglion cell (RGC) axons severed from their soma were still able to respond to guidance cues in a normal way (Harris et al., 1987). More recent findings uncovered that local protein turnover is important (Martin, 2004). Canonical Wnt signaling mediating axon guidance by regulating gene expression seems unlikely therefore. Furthermore, $Lrp6^{-/-}$ mice show no axon guidance defects, at least concerning the growth of spinal cord commissural axons (Lyuksyutova et al., 2003). However, one should be cautious with this interpretation since $Lrp6^{-/-}$ mice have spinal cord patterning defects and Lrp5 could compensate for a putative function in axon guidance. In the chick Lrp5 and Lrp6 are not expressed in commissural neurons (Wacker and Stoeckli, unpublished observation). Graef and colleagues showed that Netrin induced neurite outgrowth is mediated by the Calcineurin/NF-AT pathway (Graef et al., 2003), a cascade shared with the Wnt/calcium signaling pathway. Whether NF-AT transcription factors are required for postcommissural axon growth is not known. In C.elegans, there is evidence for canonical Wnt signaling in axon guidance. Loss of function in β-catenin/bar-1 and TCF/pop-1 causes antero-posterior (AP) axon guidance defects in D-type motor neurons (Maro et al.,

2009). The authors claim that the phenotype is at least partially due to canonical Wnt pathway. A similar situation is found in the AP guidance of C.elegans QL neuroblasts. Wnt/EGL-20 regulates posterior migration of QL neuroblast via the induction of mab-5 (a HOX gene), and therefore a change in cell fate (Maloof et al., 1999). Whether axon guidance of D-type motor neurons is also accompanied by a change in cell fate has not yet been ruled out by Maro and colleagues (Maro et al., 2009). A bifurcation of the canonical Wnt pathway was shown in cerebellar granule neurons to modulate growth cone complexity (Figure 1.6B; Salinas, 2007). Wnt7a regulates microtubule stability by inhibition of GSK3β which can phosphorylate the microtubule associated protein 1B (MAP-1B) (Lucas et al., 1998). Subsequent studies performed by the same group found that GSK3β was inhibited by Dvl (Krylova et al., 2000) and that Axin surprisingly acts upstream of Dvl and is associated with microtubules (Ciani et al., 2004). Moreover, growth cone morphology was modulated by removal of APC, a direct target for GSK3β, bound to the plus-ends of microtubules, therefore changing the direction of microtubule growth (Purro et al., 2008). In these neurons, components of the canonical pathway seem to act in a distinct arrangement: microtubule-associated Axin somehow blocks GSK3β via Dvl. APC and MAP-1B are direct targets of GSK3β which regulate microtubule dynamics and therefore growth cone morphology. It is not clear, however, how proximal events in this unusual Wnt branchlet are mediated. This divergent canonical Wnt pathway seems to regulate growth cone morphology through the modulation of microtubule dynamics but not gene expression.

Ryk, an atypical RTK, containing an extracellular Wnt-inhibitory factor (WIF) domain (He, 2004) is another putative component of the β-catenin-dependent Wnt signaling pathway. Lu and colleagues demonstrated that Wnt/Ryk/Frizzled form a ternary complex (Lu et al., 2004). Moreover, Ryk expression enhanced Wnt3a-induced TCF-Luciferase activity and downregulation was sufficient to block canonical signaling in 293 cells. Lu and colleagues created a transgenic mouse constitutively expressing a siRNA against Ryk therefore downregulating Ryk. While Wnt3a enhanced neurite outgrowth in wildtype dorsal root ganglia

(DRG) this effect was diminished in DRGs derived from Ryk siRNA mice. Whether the axon guidance phenotype was mediated by canonical Wnt signaling has not been assessed. Interestingly, in the Drosophila ventral nerve cord, anteriorly decussating commissural axons are prevented from crossing the midline through the posterior commissure by DWnt5-Derailed/Ryk signaling (Yoshikawa et al., 2003). Wouda and colleagues showed that Src family kinases act downstream of Derailed/Ryk in anterior commissural axon guidance without blocking or activating canonical signaling (Figure 1.6C; (Wouda et al., 2008)). In vertebrate development Ryk displays similar effects: i.e. in repulsion of corticospinal tract (CST) or RGC axons (Liu et al., 2005; Schmitt et al., 2006).
These data suggest that in the context of axon guidance Wnts most likely do not signal via the β-catenin-dependent pathway. However, the divergent canonical pathway governing growth cone complexity of cerebellar granule cells is an interesting candidate for microtubule rearrangements acting possibly in parallel to dynamic changes in the actin cytoskeleton. Ryk, despite its ability to activate the canonical Wnt pathway, might regulate axon growth and guidance through Wnt/Calcium and a still undefined pathway involving Src family kinases.

The Wnt/Calcium pathway is a likely candidate for the regulation of postcommissural axon guidance (Zou, 2004). Calcium signaling is well known for its function in growth cone steering and axonal elongation (Henley and Poo, 2004), first shown in Netrin-1-mediated growth cone turning of dissociated Xenopus spinal neurons (Hong et al., 2000). The release of Ca^{2+} from internal stores or its influx from the extracellular space activates a plethora of Ca^{2+}-dependent proteins (Zheng and Poo, 2007), amongst others CAMKII, Calcineurin, and protein kinase C (PKC). Calcium channels can be regulated by protein kinase A (PKA) and PKG, whose kinase activity depends on the presence of the cyclic nucleotides cAMP and cGMP, respectively (Nishiyama et al., 2003). Cyclic nucleotides are known modulators of axon guidance responses (first shown by (Song et al., 1998)). That Wnt signaling can regulate axon guidance via Ca^{2+} has already been mentioned above (Li et al., 2009). Li and colleagues

demonstrated that cortical axons are repelled and at the same time grow longer after exposure to Wnt5a microgradients. The repulsive and the growth-promoting effect were mediated by Ryk, as shown after perturbation of Ryk function with function-blocking antibodies or siRNA. The authors suggested that the repulsive effect was probably mediated in cooperation with a Frizzled receptor, since Sfrp2 was able to block repulsion but not growth. Both the growth promoting and the repulsive functions were mediated by Ca^{2+} signaling. However, while axon outgrowth seemed to be depending on IP3 receptors (releasing Ca^{2+} from intracellular stores) and transient receptor potential (TRP) channels (regulate influx of Ca^{2+} from the extracellular space), repulsion only depended on TRP channels.

Rodriguez and colleagues showed that retinal ganglion cell (RGC) axons are guided by Frz2-mediated signaling (Rodriguez et al., 2005). Unexpectedly, Sfrp1 was suggested to be the ligand activating signal transduction (Figure 1.6B). Importantly, growth cone responses upon Sfrp1 stimulation were sensitive to the G-protein inhibitor pertussis toxin and modulators of cyclic nucleotide signaling. These findings imply that RGC axons are steered by a transduction cascade reminiscent of Wnt/Calcium signaling. It appears that Calcium signaling is a common theme in axon guidance employed by Wnts and classical guidance cues alike.

Does Wnt/PCP guide commissural axons? The process of cell polarization by Wnt/PCP causes local changes in cytoskeleton, for instance the distal outgrowth of a wing hair in Drosophila, and is mechanistically similar to the process of axon guidance and cell migration. In PCP signaling cytoskeleton dynamics is regulated via the activation of Rho family GTPases (Schlessinger et al., 2009). Rho GTPases RhoA, Rac1, and Cdc42 were first identified as regulators of migrating fibroblasts in vitro (Hall, 1998), and subsequently appreciated as important factors in axon guidance (Dickson, 2002). In fibroblasts, RhoA activation causes assembly of stress fibers (contractile actin myosin filaments) and focal

adhesions, while Rac1 and Cdc42 activation induces the formation of lamellipodia and filopodia, respectively, generating protrusive forces.

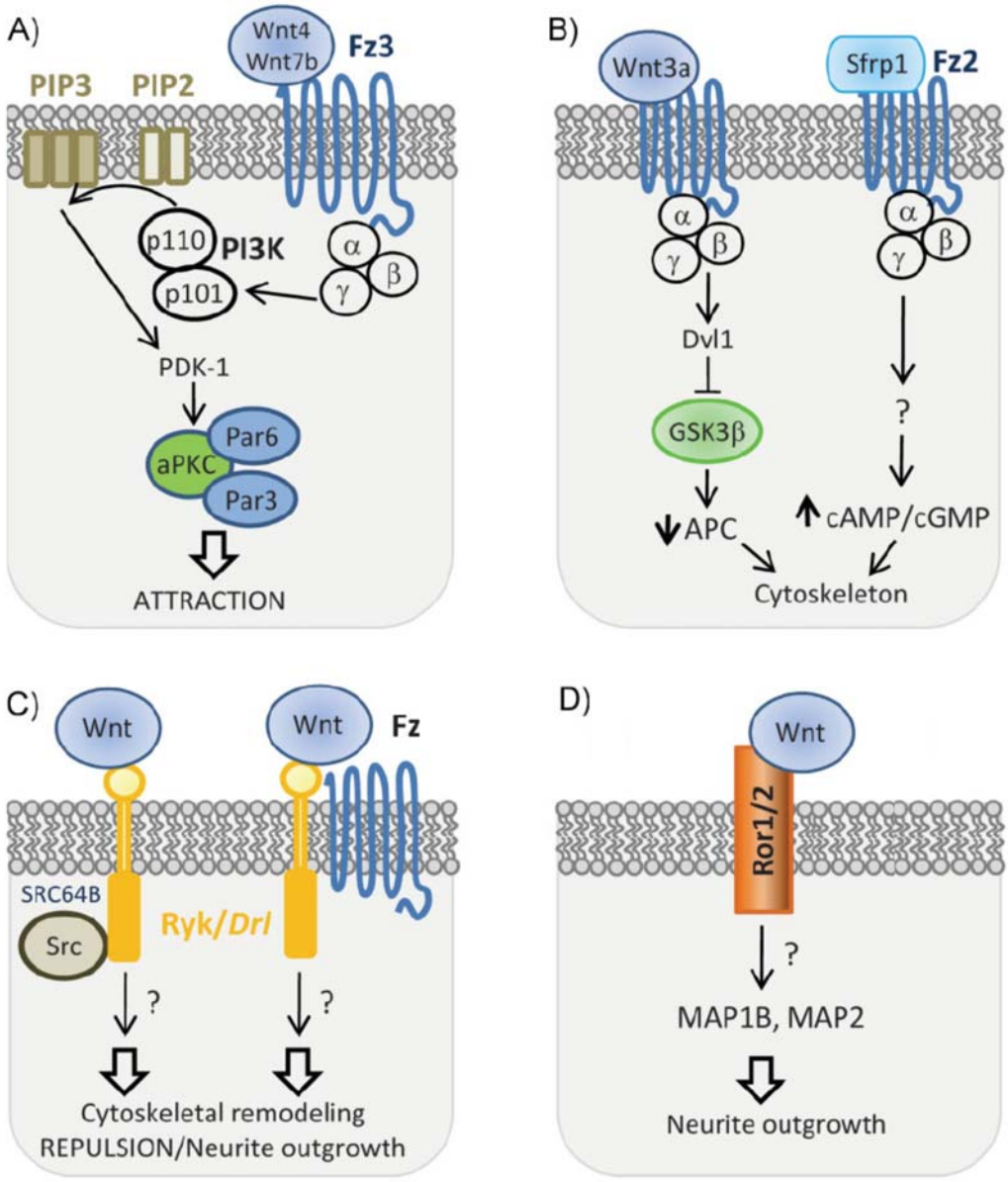

Figure 1.6 Wnt pathways in neuronal development (adapted from Sanchez-Camacho and Bovolenta, 2009).

Similar effects are seen in growth cones upon activation of Rac1 and Cdc42. RhoA activation, however, induces retraction of neurites (Hall, 1998; Jaffe and Hall, 2005). Studies on the migration of facial (nVII) motor neurons in zebrafish

and mice revealed that Wnt/PCP components are involved in neuronal migration. Bingham and colleagues first showed that the Zebrafish trilobite mutant, displaying tangential migration defects in nVII neurons, is caused by a mutation in the PCP gene Vangl2 (Bingham et al., 2002). Subsequently, Scribble1, Frizzled3a, and Celsr2 were shown to be involved in this process (Wada et al., 2005; Wada et al., 2006). Scribble1 is known to function in the establishment of apical-basal polarity (Karner et al., 2006). Work on mice confirmed a conservation of the molecular mechanisms of nVII neuronal migration (Vivancos et al., 2009). Furthermore, Vivancos and colleagues provided evidence that Wnt5a (and probably Wnt7a) is an attractive cue guiding facial motor neurons through regulation of JNK and ROCK. Additional evidence for Wnt/PCP in axon guidance comes from knock-out mice. Fzd3-/- and Celsr3-/- mice display similar defects in the formation of major tracts in central nervous system (Tissir et al., 2005; Wang et al., 2002). Reminiscent of Fzd3-/- and Celsr3-/- mice, JNK1-/- mice have a disrupted anterior commissure (Chang et al., 2003). Moreover, Chang and colleagues showed that Jnk1 can regulate the stability of microtubules through phosphorylation of MAP2 and MAP1B. Whether the correlation of phenotypes is a coincidence or due to the activation of a shared PCP-like pathway is not clear yet.

Apicobasal polarity and Wnt signaling in axon guidance. In vitro studies with migrating rat astrocytes revealed that Cdc42 regulated the microtubule cytoskeleton through Par6-aPKC (Etienne-Manneville and Hall, 2001). The Par complex, containing Par3, Par6, aPKC, and Cdc42, is known for its function in the polarization of epithelia in the apical-basal axis (Karner et al., 2006). Further studies of the same group suggested that aPKC inactivates GSK3β through phosphorylation, which leads to the association of APC with the plus end of microtubules and the accumulation of Discs large (Dlg) at leading edges of the migrating cell (Etienne-Manneville and Hall, 2003; Etienne-Manneville et al., 2005). Similar studies by Schlessinger and colleagues showed that a noncanonical Wnt pathway regulates APC localization, depends on Axin and

Dishevelled function, and cooperates with Cdc42 signaling (Schlessinger et al., 2007). These studies suggest that PCP and apical-basal polarity components (i.e. Scribble, aPKC, Dgl) might interact in axon guidance. Indeed, in mice, Wnt4-mediated postcrossing commissural axon guidance is sensitive to inhibitors of aPKC (Wolf et al., 2008). Moreover, blocking of heterotrimeric G-proteins and phosphatidylinositol-3-kinase (PI3K) caused postcrossing guidance errors in vitro. These findings imply that a Frizzled receptor (possibly Frizzled3) acts as a GPCR to activate aPKC via PI3K (Figure 1.6A). However, functional data providing evidence for the involvement of Par proteins or Cdc42 have not been generated yet.

Attraction versus Repulsion

Is attraction versus repulsion a question of Frizzled versus Ryk? Indeed, it seems that Ryk/Derailed mediates repulsion, while Frizzled receptors govern attractive responses but are not excluded from repulsive actions. Growth promotion can be conveyed by Ryk/Derailed and Frizzleds alike. It seems likely that the attractive and growth promoting effect of Wnts on mouse commissural axons is mediated by Frizzled3 (Lyuksyutova et al., 2003). Similarly, two studies present evidence that RGC axons are attracted by means of a Frizzled receptor (Rodriguez et al., 2005; Schmitt et al., 2006). Rodriguez and colleagues demonstrated, however, that the response can be modulated by ECM molecules. Furthermore, facial branchiomotor neuron (FBM) migration is severely perturbed in Frz3-/- mice, while FBM neurons are attracted by Wnt5a (Vivancos et al., 2009). In contrast, Derailed/Ryk expressing axons are repelled from the posterior commissure by DWnt5 in Drosophila (Yoshikawa et al., 2003). Cortical spinal tract (CST) axons are repelled by Wnt ligands signaling through Ryk (Liu et al., 2005). Li and colleagues showed that microgradients of Wnt5a repelled cortical axons (Li et al., 2009). This effect required Ryk and probably Frizzled2. In C.elegans, however, the frizzled homologue LIN-17 was shown to be important for D-type motor neuron axon repulsion while loss of Ryk homologue LIN-18 only induced subtle

defects (Maro et al., 2009). Therefore, it is still an open question whether different receptor complexes convey distinct outcomes in terms of axonal growth or whether extracellular (i.e. ECM molecules) and intracellular (i.e. cyclic nucleotide) signaling components tune growth cone responses. Moreover, in vitro migration assays put forth that Wnt5a-induced cell migration is mediated by Ror2 (Nishita et al., 2006). Ror2 was sufficient to induce the formation of filopodia and essential for Wnt-induced filopodia formation (Figure 1.6D). Interestingly, Dvl was required for cell migration induced by Wnt5a, but not Ror2-mediated promotion of filopodia. A follow up study by the same group identified aPKC and JNK as downstream effectors of Wnt5a/Ror2-promoted migration (Nomachi et al., 2008). Moreover, the C.elegans Ror homologue CAM-1 was identified as a possible receptor for CWN-2 (Wnt5)-mediated axon guidance (Kennerdell et al., 2009).

Evolutionary perspectives

The steering of axons is accomplished by a conserved set of axon guidance cues (Dickson, 2002). The conservation goes down to specific functions. Dorso-ventral axon guidance is regulated by Netrin homologues from C.elegans to Drosophila to vertebrates (Culotti and Merz, 1998). Similarly, Wnt genes seem to have preserved their role in longitudinal axon guidance from worm to chick and mouse (Salinas and Zou, 2008). However, mechanistically there are marked differences. In C.elegans Wnt proteins form an anterior^{low} to posterior^{high} gradient (Salinas and Zou, 2008), while in the mouse Wnt transcripts were found in an opposite gradient (Lyuksyutova et al., 2003; Liu et al., 2005). Intriguingly, in the chick Wnt transcripts were found equally distributed along the antero-posterior axis (this thesis). Sfrps, Wnt antagonists, were proposed to shape a Wnt activity gradient, which in turn instructed postcrossing axons. Why these differences between chick and mouse? One simple explanation could be that the studies in different organisms focus on distinct parts of the spinal cord concerning the longitudinal axis. While studies in the mouse zoom in on commissural axons in the upper

trunk region, lumbosacral regions are elucidated in the chick. The marked difference of Wnt4 expression in mouse and chick suggest, however, that the distinct mechanisms used are of more profound nature. Alterations in the molecular mechanism might have arisen in the last ~310 million years, after the split between birds and mammals occurred (Hedges, 2002; Reisz and Muller, 2004). It would therefore be interesting to see what mechanisms guide the same population of axons in other vertebrate model systems. The graded expression of the instructive cue alone (Wnt), rather than the regulated expression of a guidance cue and a modulating factor (Sfrp), appears to be a more simple mechanism and might therefore illustrate the ancestral mode of action. On the other hand, it is not clear whether the role of Shh is conserved in the mouse (Stoeckli, 2006). A recent study suggest that, in the mouse, Shh does sensitize commissural axons to repulsive cues in the floor plate (Semaphorin3s), rather than acting directly as seen in the chick (Bourikas et al., 2005; Parra and Zou, 2009). It is likely, however, that other factors than Wnt guide postcrossing commissural axons in the mouse. Similarly, the earlier trajectory of commissural axons is governed by the conjoint action of several axon guidance systems, probably allowing the high precision needed to wire the nervous system.

In summary, Wnt proteins are important regulators of axon guidance and participate in the steering of many different neuronal populations across different species. They act as attractive as well as repulsive cues and can additionally promote neurite outgrowth. The intracellular cascades that confer Wnt-mediated growth and guidance are slowly unraveled and show, probably not so surprising, a large overlap with quite well known molecular mechanisms employed by classical axon guidance cues. Wnt/Calcium, Wnt/PCP, and an unusual cascade involving canonical Wnt components seem to participate in the modulation of the growth cone cytoskeleton. Whether these signaling pathways are part of a network that regulates axonal steering and growth or whether distinct cascades regulate different aspects of axonal behavior, i.e. attraction versus repulsion versus growth, is still elusive.

Vertebrate commissural neurons - dI1

Dorsal spinal cord neurons are derived from distinct populations of dorsal interneurons (dI1-6). The most dorsal population (dI1) is well characterized in terms of the molecular mechanisms determining their early axonal trajectory (Figure 1.7). The initial dorsoventral growth of dI1 axons is mediated by roof-plate derived chemorepellents of the BMP family (Augsburger et al., 1999). In vitro and in vivo experiments suggest that a BMP7/GDF7 heterodimer guides dI1 axons ventrally (Butler and Dodd, 2003) mediated by BMPRIB (Yamauchi et al., 2008). Additionally, the floor plate secrets long-range cues, Netrin-1 (Kennedy et al. 1994) and Shh (Charron et al., 2003), which attract dI1 axons. Netrin mediates its attractive/outgrowth promoting effect by means of DCC (deleted in colorectal cancer; (Serafini et al., 1996)). In vitro experiments using a function-blocking antibody against DCC revealed that the antibody can block DCC-mediated growth promotion but not turning (Keino-Masu et al., 1996). Subsequently, DSCAM (Down syndrome cell adhesion molecule) was identified as a receptor for Netrin-1 transducing the attractive response in concert with DCC (Ly et al., 2008). Shh attraction depends on Smoothened (Charron et al., 2003), BOC (Brother of CDO; Okada et al., 2006), and possibly Gas1 (growth arrest-specific; Allen et al., 2007). Recently, Draxin (dorsal repulsive axon guidance protein), a novel roof plate-derived axon guidance molecule, was identified and found to repel dI1 axons (Islam et al., 2009). Subsequently, adhesion molecules guide commissural axons (dI1) locally at the midline. Cell adhesion molecules of the Ig superfamily mediate contact attraction: Axonin-1/TAG-1 on the axon interacts with floor plate NrCAM to provide a positive cue that allows crossing of the midline (Stoeckli, 1998; Stoeckli and Landmesser, 1995). In the rat, Axonin-1/TAG-1 is downregulated on commissural axons after crossing (Dodd and Jessell, 1988), possibly lowering overall adhesion and preventing axons from recrossing. Chicken postcrossing-commissural axons however, still strongly express Axonin-1/TAG-1 (Stoeckli and Landmesser, 1995), revealing that other mechanisms are at work as well (e.g. the repulsive

Slit-Robo system, see below). F-spondin, an adhesion molecule of the TSR (Thrombospondin Type-1 Repeat) superfamily, is expressed in the floor plate and can promote outgrowth of commissural axons. In vivo data suggest that F-spondin regulates the stereotypic turning angle and growth along the contralateral floor plate border (Burstyn-Cohen et al., 1999). Moreover, cleavage of F-spondin generates two fragments with functionally distinct roles. A repulsive peptide enriched in the membrane of floor-plate cells, and an adhesive fragment localized to the basement membrane constrict commissural axon trajectory (Zisman et al., 2007). Gore and colleagues showed that stem cell factor (SCF or Steel factor), expressed in the floor plate, can promote the growth of postcrossing axons, probably via Kit, a receptor tyrosine kinase (Gore et al., 2008). Furthermore, commissural axons of Steel and Kit mutant mice transiently line up at the contralateral border of the floor plate. After crossing, commissural axons are prevented from recrossing by repulsive Slit/Robo signaling. The secreted ligands Slit1-3 are expressed in the floor plate (Brose et al., 1999; Long et al., 2004). The Robo receptors, mediating the repulsive Slit signals, are expressed in pre- and postcommissural axons. Nevertheless, precrossing commissural axons are insensitive to Slits as shown by in vitro experiments (Zou et al., 2000). There are, however, contradictory studies suggesting that precrossing axons are Slit responsive (Kadison et al., 2006; Mambetisaeva et al., 2005). Several mechanisms were described that prevent Slit-mediated repulsion before axons have crossed the midline. Moreover, the attenuation of attractive signals enables axons to leave the floor plate (see Axonin-1/TAG-1 above and Robo/DCC interaction below). First, expression levels of Robo receptors are kept low before and are upregulated after crossing (Chen et al., 2008b; Kidd et al., 1998; Long et al., 2004). Second, a Robo3 splice variant (Robo3.1) is highly expressed on precrossing axons and inhibits Robo1/2-mediated repulsion. After crossing Robo3.1 is down- and a second splice variant, Robo3.2 is upregulated, allowing/mediating repulsion in concert with Robo1 and Robo2 (Chen et al., 2008b). Third, Netrin attraction, but not growth promotion, is attenuated by intracellular Robo/DCC interaction upon Slit-mediated Robo activation (Stein and

Tessier-Lavigne, 2001). After crossing the midline commissural axons of the dI1 population make a sharp turn and start growing in the longitudinal axis towards the brain. Compared to the large body of literature describing dorsoventral guidance of commissural axons, little is known about guidance into the anteroposterior axis. Wnt4 was the first molecule identified in the rostral turning of axons (Lyuksyutova et al., 2003). In an in vitro experiment Lyuksyutova and colleagues demonstrated that postcommissural axons are guided in the longitudinal axis via a secreted cue. This secreted cue was then found in a family of well known glycoproteins, the Wingless/Wnt family. COS cells expressing Wnt4 were able to redirect postcrossing axons in explants, while several Wnts (Wnt1, Wnt4, Wnt5a, Wnt6, Wnt7b) displayed growth promoting effects in vitro. Wnt4 mRNA turned out to be expressed in a rostral^{high} to caudal^{low} floor plate gradient suggesting that it acts as an attractive/growth-promoting cue for postcommissural axons. Moreover, commissural axons of frizzled3 knockout mice show anteroposterior guidance defects indicating that Wnt4 might mediate its attractive effect via frz3 receptor. A follow-up study by the same group provides evidence that PI3K-aPKC signaling is required for commissural axon guidance downstream of Wnt4 (Wolf et al., 2008). In a subtractive hybridization screen for floor plate derived postcrossing guidance cues our group identified Shh (Bourikas et al., 2005). In vivo downregulation of Shh caused AP-guidance phenotypes while in vitro experiments reveal that Shh repelled commissural axons after crossing. Repulsion was not transduced by Ptch/Smo since none of them was expressed in commissural neurons and Smo-inhibitor cyclopamine had no effect on postcrossing axon guidance. Intriguingly, Hhip was transiently upregulated at the time commissural axons turn into the longitudinal axis and downregulation of Hhip phenocopied Shh loss-of-function (Bourikas et al., 2005).

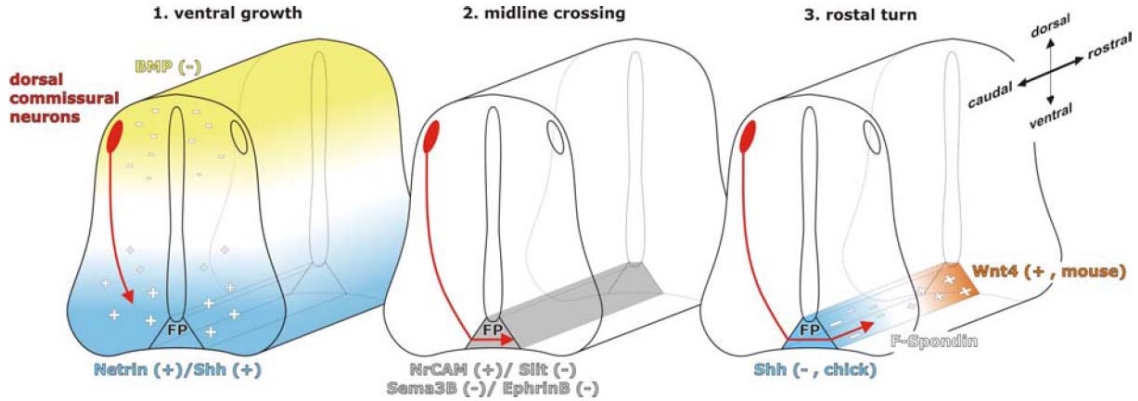

Figure 1.7 Dorsal commissural axon guidance. Dorsoventral growth of commissural axons is mediated by repulsive and attractive cues. A balance of negative and positive forces allows midline crossing, but not recrossing. After reaching the contralateral side of the neural tube, the axons are guided into the longitudinal axis by gradients of morphogens. (From Thomas Baeriswyl, 2007.)

In ovo RNAi

From spontaneous mutations to spatio-temporally controlled knockdown

In the beginning of the 20th century, at the dawn of fly genetics, scientist still relied on the rare occurrence of spontaneous mutations. Geneticists tried to develop tools to induce heritable phenotypic changes in plants and animals (Sturtevant, 1965). Only in 1927 a study by Hermann Joseph Muller convincingly demonstrated that X-rays can be used to induce “artificial transmutations” in Drosophila (Muller, 1927). The use of radiation and chemicals led to the discovery of a plethora of mutations. Of course, such approaches, today we would call it forward genetics, are undirected and the phenotypes need to be found and the mutations mapped. It still was a long way from forward to reverse genetics (Weissmann et al., 1979). Only in 2007 the Nobel Prize was given to three scientists for their efforts to create transgenic mice by the use of stem cells (www.nobelprize.org). Today, techniques to create various specific gene modifications are available. Importantly, the knock out of a gene is still the most powerful path to understand gene function. Knock out technologies are now highly sophisticated allowing for conditional or inducible removal of a gene. However, these approaches are still time consuming and expensive and not

always feasible due to early embryonic lethality. Moreover, the temporal resolution might not be good enough for developmental studies where timing is crucial due to possible interference with earlier gene functions (Bourikas and Stoeckli, 2005; Baeriswyl and Stoeckli, 2006). The emergence of RNA interference turned the situation upside down. In 1998, Fire and colleagues published a paper where they revealed that injection of double-stranded RNA (dsRNA) showed much stronger interference in C.elegans than antisense or sense oligonucleotides (Fire et al., 1998). These groundbreaking findings were honored with the Nobel Prize only 8 years later, in 2006. In the meantime, the basic mechanisms of RNA interference were elucidated (Almeida and Allshire, 2005; Hannon, 2002). Double-stranded RNA applied to a cell is recognized and cleaved by a helicase/RNase III-like enzyme called Dicer. The resulting ~23bp small-interfering RNAs (siRNAs) are incorporated into the RNA-induced silencing complex (RISC). RISC unwinds the double-stranded siRNA, uses it as a template to sequence-specifically bind to and subsequently degrade an mRNA (Figure 1.8). Today the range of application for RNAi has widened from C.elegans to other invertebrates and vertebrates, like mouse or chick.

The chick, in ovo electroporation and RNAi

The chick has always been a favored model organism for developmental biology (Davey and Tickle, 2007; Stern, 2005). It is a very robust vertebrate and as an oviparous animal develops ex utero, allowing easy access during ontogeny. Before sophisticated genetic tools were available for vertebrates, the chicken embryo was preferably used to reveal developmental mechanisms, mainly by grafting and transplantation experiments. Maturation and differentiation of B cells (Reynaud et al., 1987), limb and neural crest cell development (Le Douarin and Teillet, 1973; Summerbell and Lewis, 1975) are only a few examples where the chicken embryo helped to unravel basic biological mechanisms. Overexpression by delivery of DNA became possible with the adaption of in vitro transfection methods to in vivo studies. Lipofection, retroviruses, and electroporation were

successfully used to study gene function (Ishii et al., 2004; Muramatsu et al., 1997). However, loss-of-function experiments were only feasible when a dominant-negative version of a gene was available. This limitation was overcome by the combination of in ovo electroporation and RNA interference (Baeriswyl, 2006; Bourikas and Stoeckli, 2003; Pekarik et al., 2003).

In ovo RNAi

In ovo RNAi, injection of dsRNA into the chicken embryo and subsequent in ovo electroporation to transfect cells with the applied nucleic acid, is a fast and cheap method to produce lof phenotypes in a developing avian embryo. The first step is the production of an appropriate dsRNA to target the desired mRNA for knock down. Several forms of duplex RNA have previously been used and shown to be potent in downregulating the target protein: long dsRNA, shRNA (short hairpin RNA), and siRNA (Baeriswyl and Stoeckli, 2008; Bourikas et al., 2005; Chesnutt and Niswander, 2004; Dai et al., 2005; Das et al., 2006; Katahira and Nakamura, 2003; Pekarik et al., 2003; Sato et al., 2004; Stepanek et al., 2005). Short-hairpin RNAs are delivered by plasmid DNA encoding the RNA sequence in a sense-loop-antisense-oligodT configuration or embedded in a miRNA operon with an appropriate promoter (usually U6 or H1). The RNA transcript then hybridizes to a stem-loop conformation and is subsequently processed by the RNAi machinery. Production of siRNAs is usually done in vitro by digesting long dsRNA. Long dsRNA can be easily transcribed and hybridized in vitro and afterwards applied to the embryo. The only prerequisite is the availability of a fragment of your gene of interest (lengths of 400 to 2000bp are feasible). However, now with the chicken genome sequenced, this is not a problem any more. Expressed sequence tags (ESTs) are commercially available for virtually every chicken gene. By the in vitro synthesis of sense and antisense strands from an EST and subsequent hybridization of these, long dsRNA is produced very quickly, cheaply and easily.

Spatio-temporal control

In ovo RNAi enables a tight spatio-temporal control of knockdown. From E2 (embryonic day 2) up to E4 the embryo is accessible in ovo for injections into the neural tube. This time window can even be enlarged by ex ovo culture techniques (Baeriswyl and Stoeckli, 2008; Luo and Redies, 2005). The temporal flexibility of the application of the dsRNA makes it possible to induce RNAi at the time when your gene of interest starts to be expressed. The spatio-temporal expression pattern can be assessed by means of the same EST used to produce dsRNA by in vitro transcription of tagged RNA molecules. One the other hand, spatial control can be gained by varying the injection site in the longitudinal axis (from spinal cord to different brain vesicles), and, of course, is not restricted to neural tissue. Additionally, the orientation of the electrodes and the parameters used for electroporation offer further spatial restriction of transfection. The possibility of varying all these parameters (time of injection, site of injection, strength and orientation of eletroporation) make in ovo RNAi a valuable tool for the study of molecular mechanism in developmental biology.

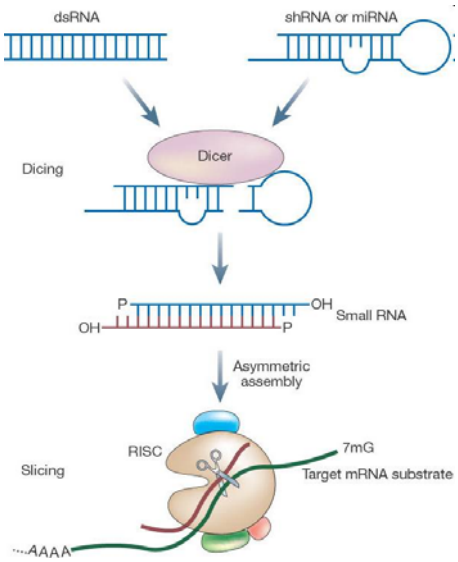

Figure 1.8 The RNAi machinery. Exogenously applied dsRNA or endogenously produced shRNA or miRNA is processed by Dicer. The resulting small RNA is incorporated into RISC and subsequently used to sequence-specifically degrade mRNA.
(http://www.welgeninc.com/technologies.html)

Aim of my thesis

In the developing chicken embryo, Shh guides dorsal commissural axons into the longitudinal axis after they have crossed the midline (Bourikas et al., 2005; Lyuksyutova et al., 2003). A similar function is covered by Wnt proteins in the mouse spinal cord (Lyuksyutova et al., 2003). It is not known whether the role of Wnt proteins is conserved between mouse and chick.

Therefore, the aim of my thesis was the functional characterization of Wnt proteins as axon guidance cues at the midline of the chicken spinal cord. Furthermore, Sfrps, known Wnt antagonists, were included into the functional analysis. More specifically, I set out to:

1. find candidate Wnt and Sfrp genes by expression analysis.
2. functionally characterize candidate Wnt and Sfrp genes in vivo and in vitro.

3. Manuscript

A Wnt activity gradient, shaped by Sfrps, guides postcommmissural axons in the chicken spinal cord

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key words: axonal pathfinding, secreted frizzled-related protein, Sfrp, chicken embryo, in ovo RNAi

ABSTRACT

After midline crossing, axons of dorsolateral commissural neurons turn rostrally into the longitudinal axis of the spinal cord. In mouse, the graded distribution of Wnt4 attracts post-crossing axons rostrally. In contrast, in the chicken embryo, the graded distribution of Shh (Sonic hedgehog) guides post-crossing axons by a repulsive mechanism mediated by Hedgehog-interacting protein. Based on these observations we tested for a possible cooperation between the two types of morphogens. Indeed, we found that Wnts also act as axon guidance cues in the chicken spinal cord. However, in contrast to the mouse, *Wnt* transcription did not differ along the anteroposterior axis of the spinal cord. Rather Wnt function was regulated by a gradient of the Wnt antagonist Sfrp1 (Secreted frizzled-related protein1). We therefore suggest that a Wnt activity gradient, in cooperation with Shh, guides postcrossing commissural axons into the longitudinal axis.

INTRODUCTION

The molecular mechanisms that guide commissural axons towards the floor plate, the ventral midline of the spinal cord, have been relatively well characterized. Initially, commissural axons are repelled by BMP7/GDF7 heterodimers and Draxin derived from the roof plate (Augsburger et al., 1999; Butler and Dodd, 2003; Yamauchi et al., 2008; Islam et al., 2009). At the same time they are attracted towards the floor plate by Netrin and Shh (Kennedy et al., 1994 and 2006; Serafini et al., 1996; Charron et al., 2003). While these long-range guidance cues determine the direction of growth, they do not specify the exact pathway of the axons. This is done by short-range guidance cues, such as Axonin-1 and NgCAM, two molecules of the immunoglobulin superfamily of cell adhesion molecules (Stoeckli and Landmesser, 1995). Due to the interaction of Axonin-1 on the surface of commissural growth cones with NrCAM expressed by floor-plate cells, axons cross the midline before they turn rostrally along the longitudinal axis (Stoeckli and Landmesser, 1995; Stoeckli et al., 1997). Slits and their receptors, the Robos, counteract the attractive effect mediated by Axonin-1 and NrCAM, and therefore, are important for the exit of commissural axons from the floor plate ((Long et al., 2004; Sabatier et al., 2004); Philipp et al., submitted). F-spondin contributes to the stereotypic turning angle of commissural axons into the longitudinal axis, without affecting the direction of turning (Burstyn-Cohen et al., 1999).

More recently, guidance molecules for post-crossing commissural axons have been identified. These molecules belong to the family of morphogens and control the axonal pathfinding of different types of neurons (Bovolenta, 2005; Ciani and Salinas, 2005; Salinas, 2003; Sanchez-Camacho et al., 2005). In the mouse, an effect of Wnt4 was demonstrated in explant cultures (Lyuksyutova et al., 2003). In the chicken embryo, we identified a different morphogen, Sonic hedgehog (Shh), as an axon guidance cue for post-crossing commissural axons both in vivo and in vitro (Bourikas et al., 2005). In agreement with its graded expression in the

mouse floor plate, Wnt4 was found to act as an attractant. High *Wnt4* mRNA expression was found in the floor plate at rostral levels and low expression at more caudal levels (Lyuksyutova et al., 2003). In contrast, Shh was found to act as a repellent for post-crossing axons, consistent with its expression pattern characterized by high levels of *Shh* mRNA and protein in the caudal floor plate and low levels more rostrally (Bourikas et al., 2005). Thus, Shh has a dual role in commissural axon guidance. First, it acts as a chemoattractant in parallel to Netrin-1 (Charron et al., 2003), then, only a few hours later, Shh switches from attractant to repellent and pushes post-crossing axons rostrally (Bourikas et al., 2005). This change in activity is possible due to a switch in receptors. While pre-commissural axons are growing towards the floor plate, they are attracted by Shh mediated by Smoothened (Smo) (Charron et al., 2003) and Boc (Okada et al., 2006). After reaching the midline, they no longer express Ptc and Smo but use Hedgehog-interacting protein (Hhip) as the receptor that mediates the repulsive response to Shh (Bourikas et al., 2005).

These findings link the two families of morphogens to axon guidance and raise the question whether Wnts and Shh would cooperate in post-crossing commissural axon guidance (Stoeckli, 2006). To address this issue, we explored the expression patterns of *Wnts* and *Secreted frizzled-related proteins (Sfrp),* known Wnt antagonists, in the embryonic chicken spinal cord. Interestingly, we found no evidence for a *Wnt* expression gradient in the chicken spinal cord. Rather *Sfrp1* expression showed a strong rostral low to caudal high gradient. Functional analysis by loss and gain of function experiments demonstrated that Wnt5a, Wnt7a, and Sfrp1, but not Wnt4 are involvement in axon guidance along the longitudinal axis of the lumbosacral spinal cord. In vitro experiments confirm guidance functions for Wnts and show that Sfrp1 acts indirectly by regulating Wnt activity. Our results suggest that a Wnt activity gradient, shaped by the Wnt antagonist Sfrp1, guides postcrossing commissural axons in vivo and that Wnts cooperate with Shh (Bourikas et al., 2005) in the chick. This is the first study

showing that an activity gradient shaped by an antagonist, rather than by diffusion or expression, guides axons in developing tissue.

RESULTS

Several *Wnts* are expressed in the floor-plate area of the embryonic chicken spinal cord

Based on the identification of Wnt4 as a guidance cue for post-crossing commissural axons in the mouse spinal cord (Lyuksyutova et al., 2003), we analyzed its expression pattern in the chicken spinal cord during the time when axons of dorsolateral commissural neurons cross the floor plate and turn into the longitudinal axis. At the lumbosacral level of the spinal cord, axons have reached the floor-plate area at HH22 (stage 22 according to Hamburger and Hamilton, 1951). At HH24 they turn into the longitudinal axis (Bourikas et al., 2005). During this time window, *Wnt4* was expressed at high levels in the dorsal spinal cord but only at low levels in a small, narrow expression domain in the ventral spinal cord (Figure S1). Unlike in the mouse, *Wnt4* was not detectable in the chicken floor plate. Based on published expression patterns (Fokina and Frolova, 2006; Hollyday et al., 1995), *Wnt1*, *Wnt2*, *Wnt3a*, *Wnt6*, *Wnt9a/b*, *Wnt16* could be excluded as well. Our own expression analysis by in situ hybridization excluded *Wnt5b*, *Wnt7b*, *Wnt8a/c*, *Wnt9b*, and *Wnt11* (not shown and Figure S1).
Wnt5a and *Wnt7a* were expressed in a spatial and temporal pattern that was compatible with a role in post-crossing commissural axon guidance (Figure S1). *Wnt5a* was found in the floor plate at both HH22 and HH24. *Wnt7a* was expressed adjacent to the floor plate in the area where postcommissural axons turn into the longitudinal axis.

Interference with Wnt5a and Wnt7a expression results in rostro-caudal pathfinding errors of post-crossing commissural axons

To assess a possible function of Wnts in post-crossing commissural axon guidance we used in ovo RNAi (Pekarik et al., 2003). We injected dsRNA derived from *Wnt4*, *Wnt5a*, *Wnt7a*, or *Wnt11* into the central canal of the spinal cord of

E3 chicken embryos (see Experimental Procedures for details, Figure S2). Co-injection of a plasmid encoding EGFP was used to monitor the efficiency of nucleic acid transfer into the floor plate area (Bourikas et al., 2005). As a negative control, we used dsRNA derived from *Wnt11,* which was not expressed in the spinal cord (Figure S1). Because antibodies for the targeted Wnts are not available, we assessed the specificity of downregulation by in situ hybridization (Figure S3). In ovo RNAi resulted in a specific reduction of *Wnt* mRNA levels between 29 and 39% but did not change the patterning of the neural tube or interfere with commissural axons growth towards the floor plate (Annex1). Moreover, the expression of Shh and HNF3beta in the floor plate was unaffected (Annex2).

Upon downregulation of Wnt5a and Wnt7a post-crossing axons failed to turn or made aberrant caudal turns as revealed by DiI tracing of dorsal commissural axons at the lumbosacral level of the spinal cord (Figure 2.1). Many axons did not reach the contralateral floor-plate border in the absence of Wnt5a (Figure 2.1C). This was not due to a delay of axon growth or a decreased growth rate, as axons in experimental and control embryos reached the floor plate at the same time. Furthermore, axons were still stuck in the floor plate when embryos lacking Wnt5a were analyzed at older stages (Annex3).

To quantify the severity of the observed defects caused by downregulation of Wnt5a or Wnt7a each injection site was classified as strong or no phenotype. A strong phenotype meant that more than 50% of the axons stalled before reaching the contralateral floor-plate border, or that fibers turned caudally along the longitudinal axis of the spinal cord. Caudal turns of dorsolateral commissural axons were never seen in control embryos. Using these criteria, a strong phenotype was found at 34.9±7.2% of the injection sites in embryos lacking Wnt5a and at 27.9±8.1% of the injection sites in embryos lacking Wnt7a (Figure 2.1E). In contrast, after downregulation of Wnt11 or Wnt4, the trajectories of post-crossing axons did not differ from non-injected or EGFP-expressing control embryos.

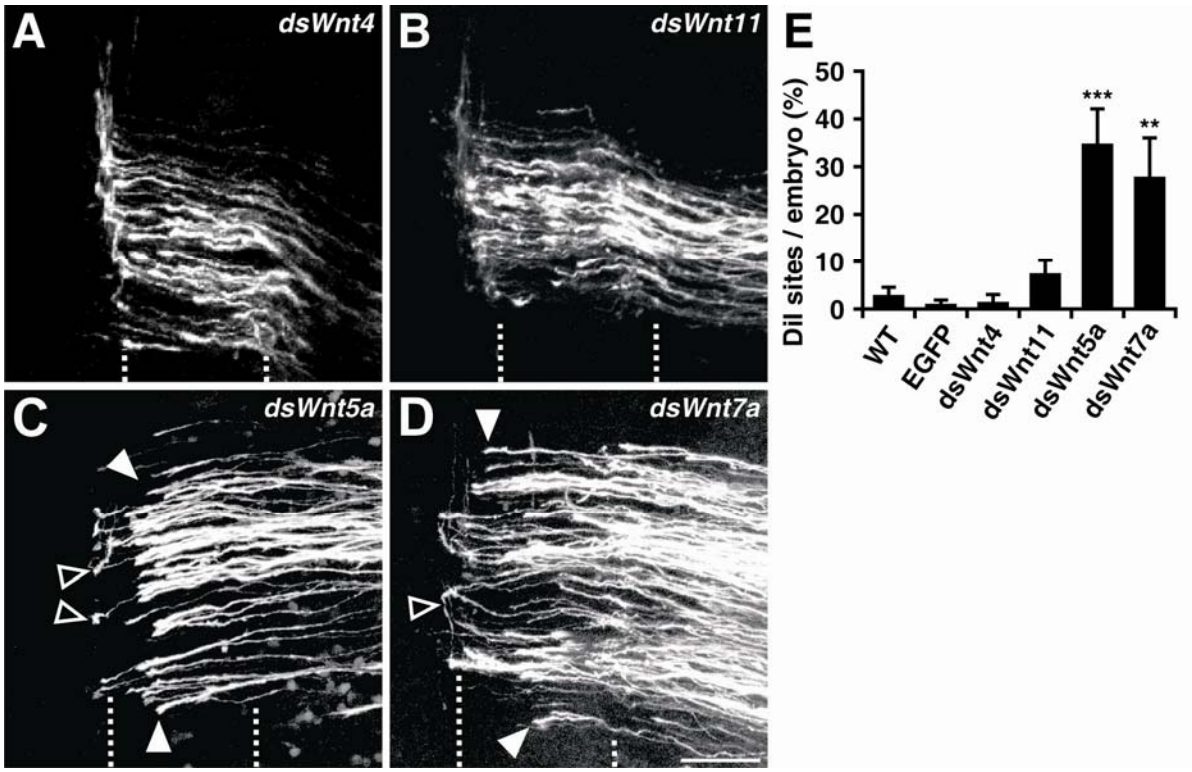


Figure 2.1 Wnt5a and Wnt7a are guidance cues for post-crossing commissural axons.
We used in ovo RNAi to downregulate Wnts in the floor-plate area. Neither *Wnt11* dsRNA (A) nor *Wnt4* dsRNA (B) injection and electroporation had an effect on post-crossing commissural axon guidance. In contrast, downregulation of Wnt5a (C) resulted in the majority of axons stalling in the floor plate (arrowheads). Those axons that had reached the contralateral floor-plate border chose randomly to turn rostrally or caudally. Axons turning caudally are marked by open arrowheads. Similarly, axons failed to turn rostrally in the absence of Wnt7a (D). In general, more axons reached the contralateral floor-plate border in the absence of Wnt7a compared to Wnt5a. Injection sites with strong phenotypes are quantified in (E). In the absence of Wnt5a a strong phenotype was observed at 34.9±7.2% of the injection sites (n=11 embryos, 112 injection sites). In the absence of Wnt7a a strong phenotype was found at 27.9±8.1% of the injection sites (n=16 embryos, 112 sites). The values found after downregulation of Wnt4 (1.5±1.5%, n=11 embryos, 67 injection sites) or Wnt11 (7.5±2.7%, n=11 embryos, 93 injection sites) were not different from controls. Untreated control embryos (3.0±1.6%, n=11 embryos, 88 injection sites) did not differ from EGFP-expressing embryos (1.1±0.8%, n=18 embryos, 107 injection sites). Values are given ±SEM. A two-tailed Student's t-test was used for statistical analysis. Three asterisks indicate $p<0.001$ for Wnt5a and two asterisks indicate $p<0.01$ for Wnt7a versus Wnt4, Wnt11, and control groups, respectively. Bar: 40 μm.

Taken together, these results indicated that Wnts were involved in rostro-caudal pathfinding of post-crossing axons in the chicken spinal cord. However, in contrast to mouse, Wnt4 did not have an effect, rather Wnt5a and Wnt7a were the Wnt family members necessary for post-crossing commissural axon guidance in the chicken embryo.

Wnts are not expressed in a rostro-caudal gradient in the embryonic chicken spinal cord

Based on their function as guidance cues for post-crossing commissural axons in the chicken spinal cord and in analogy to observations in the mouse (Lyksyutova et al., 2003), we expected to find both *Wnt5a* and *Wnt7a* in a rostral^{high} to caudal^{low} gradient.

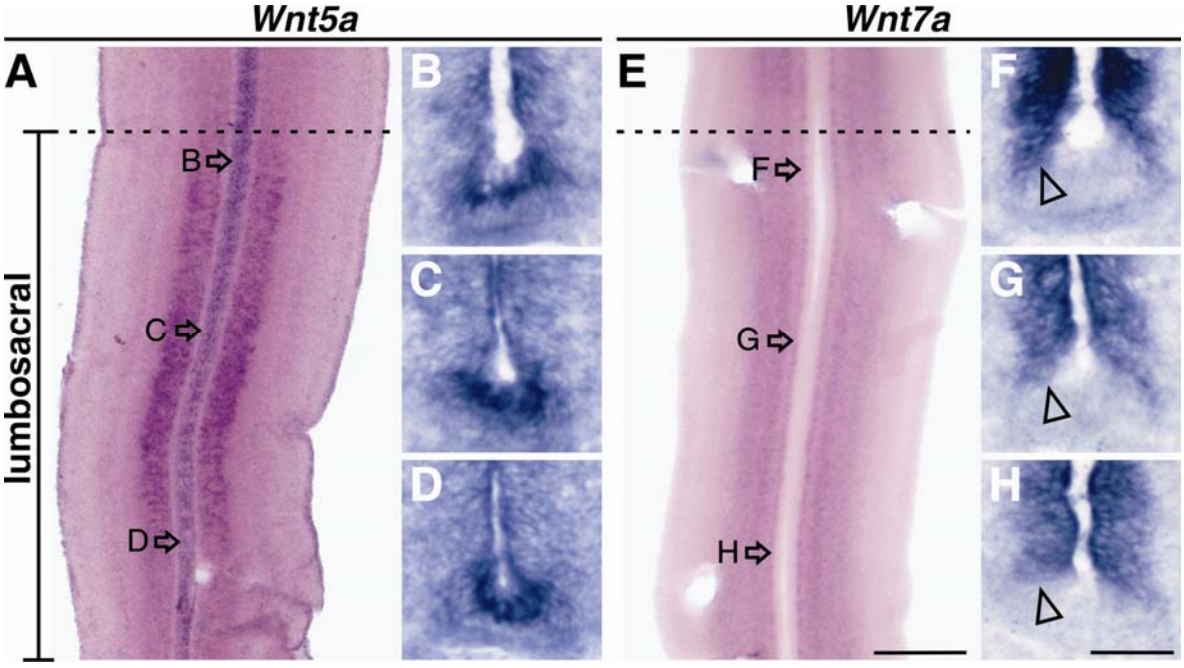


Figure 2.2 Neither *Wnt5a* nor *Wnt7a* is expressed in a gradient along the anteroposterior axis of the lumbosacral spinal cord.
Open-book preparations (A,E) and transverse sections (B-D, F-H) of HH26 spinal cords were used for in situ hybridization analyses of *Wnt5a* (A-D) and *Wnt7a* (E-H) expression. No clear gradient was detectable for *Wnt5a* mRNA in the floor plate of the lumbosacral region (see Figure S4 for quantification). Note that slightly higher levels are found at the thoracic level (not shown) and that expression of *Wnt5a* in motoneurons is restricted to lumbosacral levels of the spinal cord. No gradient was observed for *Wnt7a* in the area adjacent to the floor plate (open arrowhead in F-H). Arrows in A,E indicate levels of transverse sections shown in B-D and F-H, respectively. Bar: 50 µm for transverse sections and 500 µm for A,E.

Surprisingly, neither *Wnt5a* nor *Wnt7a* mRNA was found to be expressed in a gradient in the lumbosacral spinal cord (Figure 2.2 and S4). For the quantification we used both transverse sections taken from different levels (not shown) and open-book preparations of the lumbosacral spinal cord.

Sfrps are expressed in a rostro-caudal gradient in the embryonic chicken spinal cord

To find an explanation for the apparent contradiction between functional data and *Wnt* expression patterns, we turned to Sfrps. Addition of exogenous Sfrp was shown to antagonize Wnt activity on post-crossing commissural axons in cultures of mouse spinal cord explants (Lyuksyutova et al., 2003). Four *Sfrp* family members have been found in the chicken genome. In situ hybridization analysis indicated that three of the four *Sfrps* were expressed in the developing spinal cord between HH22 and HH26 (Figure 2.3). *Sfrp1* was expressed in the floor plate at high levels (Figure 2.3A and D) and most intriguingly exhibited a strong gradient along the longitudinal axis (Figures 2.3G and S4). However, based on their expression patterns, a role for *Sfrp2* and *Sfrp3* in post-crossing axon guidance would also be possible. *Sfrp2* was expressed dorsal to the floor plate and in the ventral ventricular zone at both HH22 (Figure 2.3B) and HH24 (Figure 2.3E). *Sfrp3* was expressed more widely in the spinal cord at HH22 (Figure 2.3C) but decreased considerably thereafter. By HH24 expression was very low in the ventricular zone except for the area adjacent to the floor plate (Figure 2.3F). *Sfrp4* was not expressed in the neural tube (data not shown). *Sfrp2* (Figures 2.3H and S4) showed a similar but less pronounced gradient compared to *Sfrp1*. *Sfrp3* (Figure 2.3I) appeared to be expressed uniformly along the rostro-caudal axis. Based on their expression pattern, Sfrps made good candidates for regulators of Wnt activity in rostro-caudal guidance of post-crossing axons.

Loss of Sfrp1 function results in rostro-caudal pathfinding errors of post-crossing axons

To test for a role of Sfrps as antagonists of Wnt5a and Wnt7a in post-crossing axon guidance, we turned again to in ovo RNAi. Specific downregulation of Sfrp1

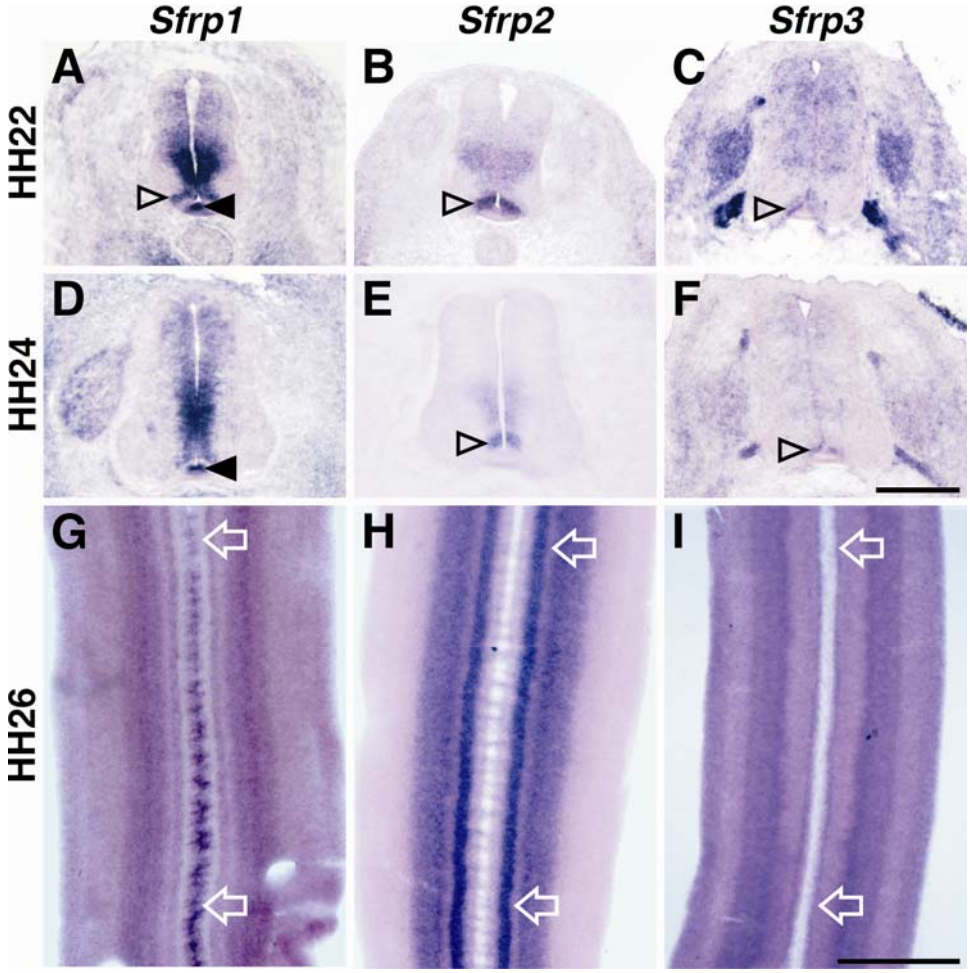


Figure 2.3 Expression patterns of *Sfrps* in the embryonic chicken spinal cord.
Sfrp1 (A,D,G) is expressed in the floor plate (arrowhead), in the ventricular zone, and in an area dorsolateral to the floor plate (open arrowhead) at HH22 (A), HH24 (D), and HH26 (G). *Sfrp2* expression (B,E,H) was found in the ventral ventricular zone with an area of stronger expression dorsal to the floor plate (open arrowhead). *Sfrp3* is expressed in an area adjacent to the floor plate (open arrowhead in C and F; I) similar to *Wnt7a*. In contrast to *Wnts* a strong gradient of *Sfrp1* was found in the floor plate along the anteroposterior axis with high levels in the caudal floor plate (G and S6). *Sfrp2* and *Sfrp3* were not expressed in a gradient along the anteroposterior axis. Compare expression indicated by arrows in G-I (see Figure S4 for quantification). Rostral is to the top. Bar: 200 µm in A-F, 500 µm in G-I.

(Figure S5) reproduced the loss-of-function phenotypes seen after silencing of *Wnt5a* and *Wnt7a* (Figure 2.4). In the absence of Sfrp1, post-crossing axons turned caudally or stalled in the floor plate at 27.5±5.2% of the DiI injection sites (Figure 2.4). Downregulation of Sfrp2 had a similar but weaker effect. Axons stalled or turned caudally at 18.3±6.7% of the injection sites. Downregulation of Sfrp3 and Sfrp4 did not affect commissural axon guidance since strong phenotypes were observed at only 10.4±4.8% and 12.2±3.0%, respectively, compared to 11.1±5.0% in untreated controls. These results were consistent with

our hypothesis that graded Wnt activity that attracted post-crossing axons rostrally was shaped by the graded expression of Sfrp1. However, based on these in vivo results an additional direct role of Sfrps on post-crossing axons could not be excluded.

Sfrp1 blocks the attractive effect of Wnt5a and Wnt7a on post-crossing commissural axons

To distinguish between a direct and an indirect role of Sfrp1 on post-crossing axons we turned to in vitro assays. Post-crossing commissural axons extended into the collagen matrix when spinal cord explants were cultured with the floor plate attached (Figure 2.5). The identity of dorsal commissural axons was confirmed by their expression of MARCKS-EGFP under the control of the Math1 promoter (Annex4). There was no difference in neurite growth when Sfrp1 was added (Figure 2.5E and H). Post-crossing axons were significantly longer than controls when explants were cultured with COS cells expressing Wnt5a (Figure 2.5B,H) or Wnt7a (Figure 2.5C,H). The growth-promoting effect of both Wnt5a and Wnt7a was blocked in the presence of Sfrp1 in the medium (Figure 2.5F-H). Pre-crossing commissural axons extending from spinal cord explants did not respond to Wnt5a or Wnt7a (Annex5).

Our in vitro results excluded a direct effect of Sfrp1 on post-crossing axons and confirmed our in vivo observations which suggested that an attractive effect of higher Wnt levels in the rostral spinal cord was generated by a graded expression of the Wnt antagonist Sfrp1. Additional evidence supporting this hypothesis was found in another in vitro assay where post-crossing commissural axons were exposed to rostral or caudal floor-plate explants, respectively (Figure 2.6). Rostral floor-plate explants were more potent than caudal floor plate in promoting growth of post-crossing axons (Figure 2.6B-E). As expected based on

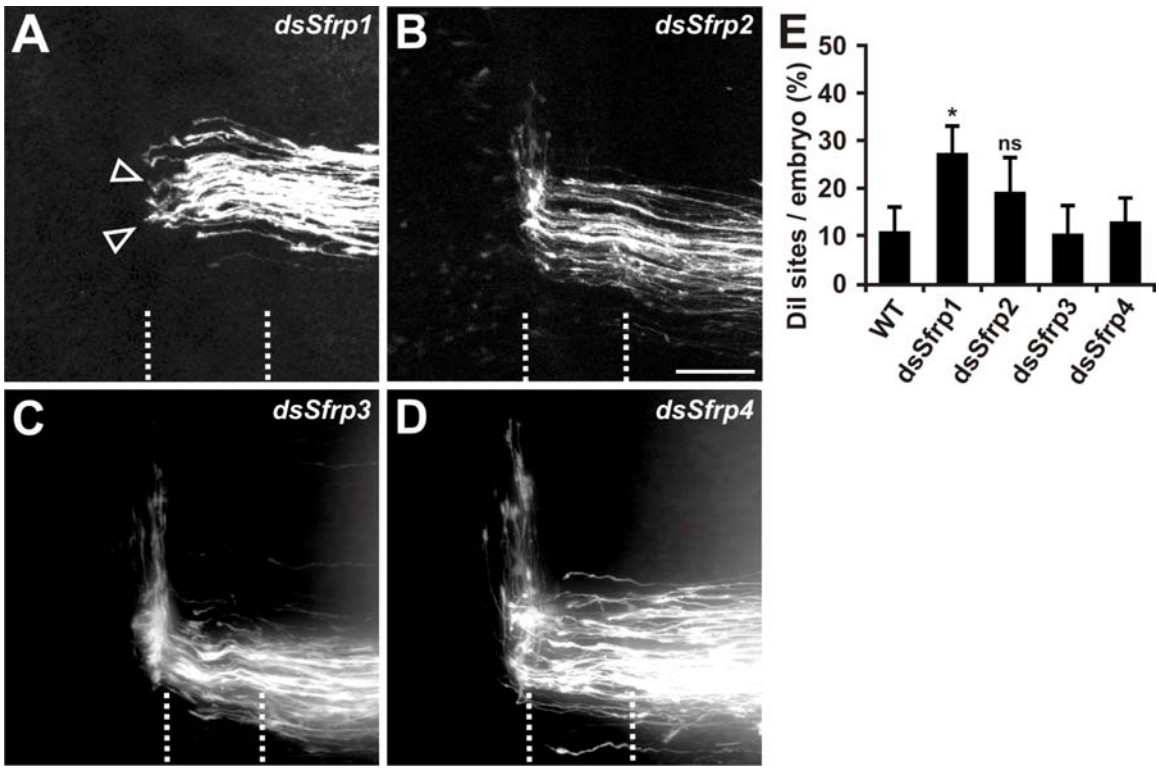


Figure 2.4 Downregulation of Sfrp1 interfered with rostro-caudal post-crossing axon guidance.
Injection and electroporation of dsRNA derived from *Sfrp1* (A), *Sfrp2* (B), but not Sfrp3 (C) or *Sfrp4* (D) interfered with the correct rostral turning of post-crossing axons along the contralateral floor-plate border. In the absence of Sfrp1 many axons failed to cross the floor plate (indicated by dashed lines) and mostly failed to turn into the longitudinal axis (A). Occasionally, caudal turns were observed (open arrowheads in A). Rostral is to the top. Strong phenotypes were observed at 27.5±5.2% of the injection sites in embryos lacking Sfrp1 (E; n=26 embryos). Fewer axons turned caudally when Sfrp2 was downregulated (B,E), as this phenotype was only seen at 18.3±6.7% of the injection sites (n=17 embryos). The value for Sfrp2 was not significantly different (ns) from values for Sfrp3, Sfrp4, and controls. The rostral turning of post-crossing axons was not affected in the absence of either Sfrp3 (10.4±4.8% strong phenotype, n=20 embryos) or Sfrp4 (12.2±3.0% strong phenotype, n=19 embryos). These values were not different from control embryos (WT), where 11.1±5.0% of the injection sites exhibited a strong phenotype (n=19 embryos). For statistical analysis the two-tailed Student's t-test was used. Values are given ±SEM. $p<0.05$ (asterisk) for ds*Sfrp1* compared to control, ds*Sfrp3*, and ds*Sfrp4*. Bar: 60 µm.

our previous assays this effect could be blocked by Sfrp1 that was added to the culture medium (Figure 2.6F-I) suggesting that the effect of floor plate explants is due to Wnt protein function.

Taken together, these experiments strongly supported our hypothesis that a graded activity of Wnts was achieved along the rostrocaudal axis of the embryonic chicken spinal cord by a graded expression of the Wnt antagonist Sfrp1.

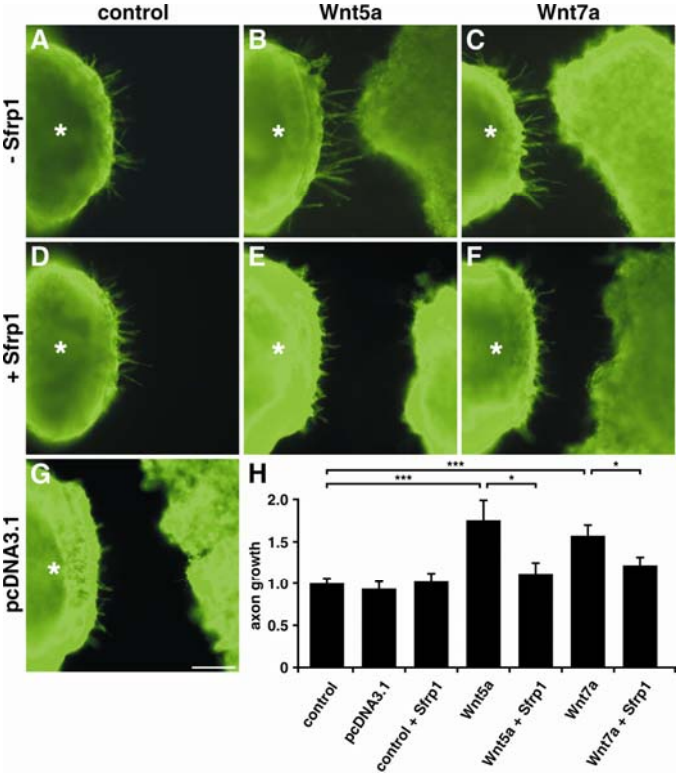


Figure 2.5 Sfrp1 blocks the attractive effect of Wnt5a and Wnt7a on post-crossing commissural axons.
Explants of post-crossing commissural neurons were cultured with or without COS cells expressing Wnt5a (B,F) or Wnt7a (C,G). COS cells transfected with the empty pcDNA3.1 vector had no effect on commissural axon growth (D, 0.94±0.08%, n=27) compared to control explants cultured without COS cells (A, 1±0.06%, n=31). In contrast, the presence of Wnt5a (B) and Wnt7a (C) considerably increased commissural axon growth (1.76±0.24, n=21, and 1.57±0.13, n=23, respectively). Adding 1µg/ml Sfrp1 had no effect on post-crossing commissural explants (E, 1.02±0.10, n=19). However, the presence of Sfrp1 significantly decreased the growth-promoting effect of Wnt5a (F, 1.12±0.13, n=20) and Wnt7a (G, 1.22±0.1, n=24), respectively. For statistical analysis the two-tailed Student's t-test was used (H). Values are given ±SEM. $p<0.001$ (3 asterisks) for Wnt5a and Wnt7a compared to control, $p<0.05$ (asterisk) for Wnt5a and Wnt7a compared to Wnt5a + Sfrp1 and Wnt7a + Sfrp1, respectively. Bar: 200 µm.

Overexpression of Wnts and Sfrps reverses the functional Wnt gradients and causes aberrant behaviors of post-crossing axons

To provide further experimental evidence for our hypothesis in vivo we carried out gain-of-function experiments. We selectively overexpressed either *Wnt5a* or *Wnt7a* in the caudal spinal cord (Figures 2.7 and S6). Spatially controlled ectopic expression of Wnts at caudal levels was expected to exceed the capacity of the endogenous Sfrps to block Wnt function caudally (Figure 2.7A). Conversely, overexpression of Sfrps at rostral levels was expected to disrupt the functional

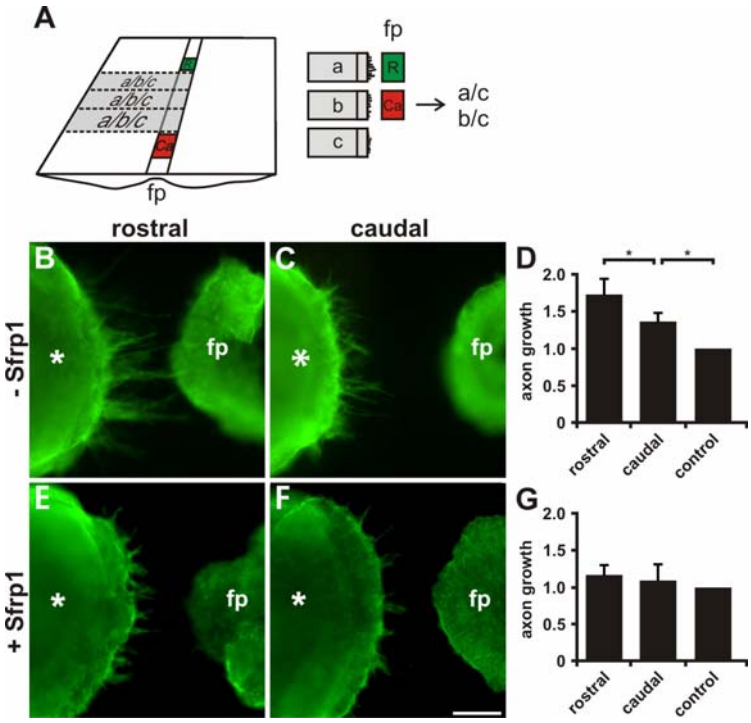


Figure 2.6 Post-crossing commissural axons are guided by higher Wnt activity in the rostral compared to the caudal floor plate.
Explants of post-crossing commissural neurons were cultured with floor-plate explants (fp) taken from either rostral or caudal lumbosacral levels (A). Three explants containing commissural neurons and two floor-plate explants dissected from a single open-book preparation were cultured as depicted. Neuronal explants were randomly assigned to one of the three conditions a, b, or c. For quantification neurite growth obtained under conditions a and b were normalized to the control condition c from the same embryo (set as 1.0, n=22). Both the presence of a rostral (B; 1.79±0.19, n=22) and a caudal (C; 1.28±0.14, n=19) floor-plate explant had a significant effect on post-crossing commissural axon growth compared to the control condition (D). However, the growth-promoting effect of rostral fp explants was significantly stronger compared to caudal fp (E). The growth-promoting effect of the fp explants could be blocked by addition of Sfrp1 (1µg/ml; F-I; rostral fp 1.17±0.26, n=10; caudal fp 1.04±0.12, n=7; control set as 1.0, n=10). For statistical analysis the two-tailed Student's t-test was used. Values are given ±SEM. $p<0.05$ (asterisk) for rostral fp compared to caudal fp, $p<0.05$ (asterisk) for caudal fp compared to control. Bar: 200 µm.

Wnt gradient by excessive levels of the antagonist (Figure 2.7B). In agreement with these predictions, post-crossing axons randomly chose to turn in either rostral or caudal direction, or they stalled at the exit site of the floor plate when Wnt5a was selectively overexpressed at caudal levels (Figure 2.7C). Similarly, when Sfrp1 was overexpressed at thoracic and upper lumbosacral levels of the spinal cord, post-crossing axons turned caudally at upper lumbosacral levels and mostly stalled at the floor-plate exit site at intermediate levels, whereas no change in the behavior was observed at caudal lumbosacral levels (Figure 2.7D). The same phenotypes were observed after caudal overexpression of *Wnt7a* and rostral expression of *Sfrp2*, respectively, although the effects were weaker than

those observed after ectopic expression of Wnt5a and Sfrp1. The changes in axon guidance were not due to aberrant spinal cord patterning, as overexpression of Wnts or Sfrps at HH19 did not alter spinal cord patterning or floor plate markers HNF3β and Shh (Annex2).

In contrast to the loss-of-function phenotypes (Figures 2.1 and 2.4) where individual DiI injection sites were analyzed, gain-of-function phenotypes were assessed for the entire embryo in relation to the overexpression domain monitored by EGFP expression (Figure S6). In embryos where expression profiles matched the intended overexpression pattern of Wnts or Sfrps, the DiI injection sites were analyzed for the expected behavior (Figures 2.7A,B and S6). If the direction of post-crossing axons all along the anterior-posterior axis was according to the expected pattern the embryo was scored as positive. Caudal overexpression of *Wnt5a* resulted in the expected axon growth pattern in 67% of the embryos (n=12). The effect of *Wnt7a* overexpression was much weaker and resulted in the expected phenotype in only 3/11 of the embryos. Similarly, overexpression of *Sfrp2* was less effective than *Sfrp1* overexpression with the expected pattern observed in 25% (n=12) and 50% (n=10) of the embryos, respectively. Axonal navigation after *Sfrp3* overexpression was not different from untreated control embryos with changes from the normal pattern in only one of the embryos (n=7). The repulsive activity derived from the graded expression of Shh in the caudal spinal cord explains the failure to turn rather than a complete reversal of the growth direction that was observed for most axons (Bourikas et al., 2005).

In summary, our gain-of-function experiments support a model that predicts a Wnt activity gradient that is shaped by the graded expression of the Wnt antagonists, the Sfrps, as a guidance mechanism for post-crossing commissural axons.

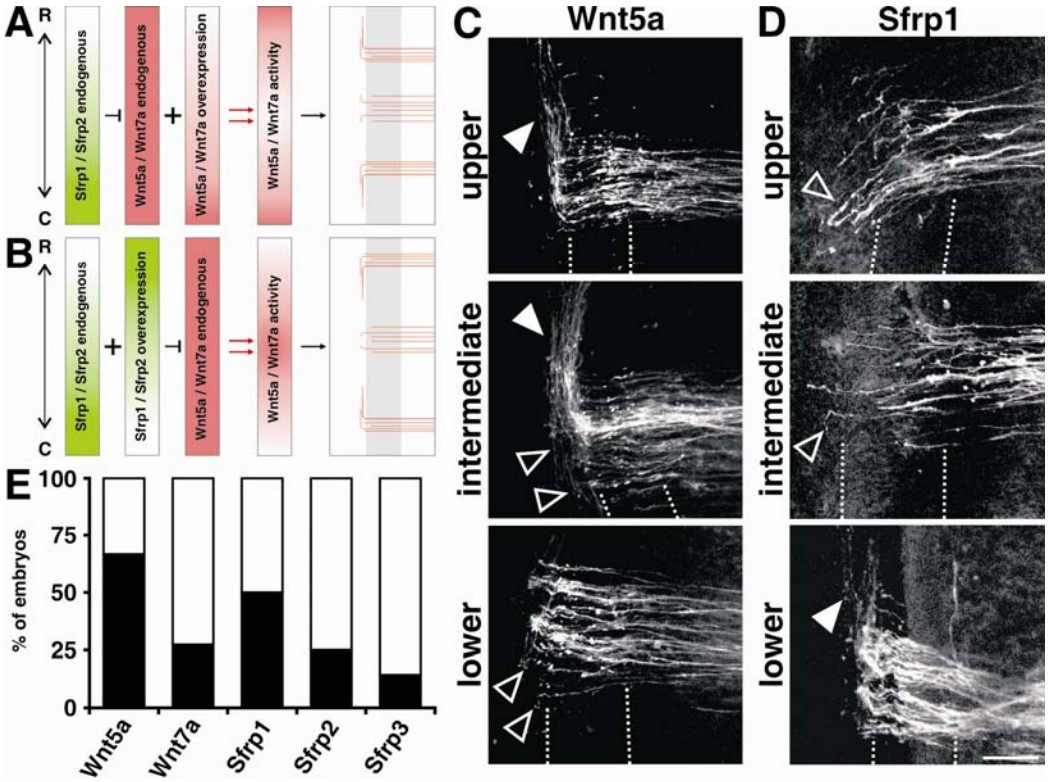


Figure 2.7 Reversal of the functional Wnt gradients by overexpression of either *Wnts* or *Sfrps* resulted in the expected anteroposterior guidance phenotypes.
To test our hypothesis that a caudal^{high} to rostral^{low} Sfrp gradient was producing a functional Wnt gradient despite the homogenous rostro-caudal expression of *Wnts*, we expressed *Wnt5a* or *Wnt7a* ectopically in the caudal spinal cord (A). This would result in a double-headed gradient with high Wnt activity levels in the thoracic (due to endogenous Wnt) and in the caudal spinal cord (due to overexpression). The expected behavior of post-crossing axons is indicated schematically. Ectopic expression of *Sfrp1* or *Sfrp2* in the thoracic spinal cord would block Wnt activity at thoracic and upper lumbosacral levels, and would therefore disrupt the Wnt activity gradient (B). In this case postcommissural axons were expected to turn correctly in the caudal but not in the more rostral lumbosacral and thoracic spinal cord. Indeed we observed the expected turning patterns in 67% of the embryos after ectopic expression of *Wnt5a* in the caudal spinal cord (C,E; see Figure S6 for an overlay of the DiI-labeled axons with EGFP expression as a means to assess ectopic *Wnt* or *Sfrp* expression). Overexpression of *Wnt7a* had a weaker effect, resulting in the expected turning pattern in 27% of the embryos. Ectopic expression of *Sfrp1* in the thoracic spinal cord resulted in the expected turning pattern in 50% of the embryos (D,E). Overexpression of *Sfrp2* had a weak effect resulting in a change in the turning pattern in 25% of the embryos (E). No effect was found after expression of *Sfrp3* (14% of the embryos exhibited changes; E). Rostral is to the top in A-D. The floor plate is indicated by dashed lines. Arrowheads indicate axons turning correctly in rostral direction. Open arrowheads indicate axons turning caudally. Bar: 60 µm.

Taken together our results suggest a model for post-crossing commissural axon guidance in the chicken spinal cord that is based on both Shh and Wnts. In contrast to the mouse, where *Wnt4* was found to be expressed in a gradient in the floor plate, with high levels rostrally and low levels caudally, Wnt4 is not involved in post-crossing axon guidance in the chick. Rather, Wnt5a and Wnt7a direct axons rostrally upon floor plate exit. However, neither *Wnt5a* nor *Wnt7a*

were found to be expressed in a gradient comparable to the one found for *Wnt4* in the mouse. Thus, in the chicken spinal cord a functional Wnt gradient rather than an expression gradient is attracting post-crossing commissural axons rostrally. The graded activity is achieved by a graded expression of the Wnt antagonist *Sfrp1*. These results suggest that axons are pushed rostrally by the repellent activity of Shh and attracted rostrally by the graded Wnt activity that is achieved by increasing blockade of Wnt activity in the caudal spinal cord (Figure 2.8).

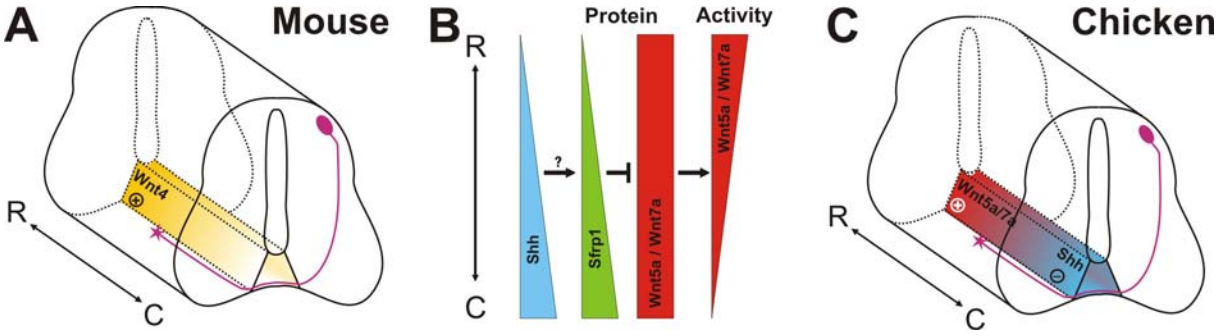


Figure 2.8 Morphogens of the Wnt family direct postcommissural axons rostrally in both mouse and chicken embryos but by a different mechanism.
A transcriptional gradient of *Wnt4* was found in the mouse floor plate (Lyuksyutova et al., 2003). High Wnt4 levels attract postcommissural axons rostrally (A). In the chicken embryo *Wnt5a* and *Wnt7a* are expressed at homogeneous levels along the rostro-caudal axis (B). However, the graded expression of *Sfrps* with high levels caudally and low levels rostrally form a functional Wnt gradient by blocking Wnt activity caudally but not rostrally. The resulting gradient of Wnt function with high activity rostrally and low activity caudally cooperates with the repellent activity of Shh that is expressed in a rostral^{low} to caudal^{high} gradient (C) to direct postcommissural axons along the longitudinal axis of the spinal cord.

DISCUSSION

In a previous study, we identified a role of the morphogen Shh in post-crossing commissural axon guidance in the chicken spinal cord (Bourikas et al., 2005). In contrast to pre-crossing commissural axon guidance, where Shh was shown to act as a chemoattractant in parallel to Netrin-1 (Charron et al., 2003; Yam et al., 2009), the activity of Shh was not mediated by Smoothened and Boc but rather by Hhip (Hedgehog-interacting protein). Consistent with its expression pattern Shh acts as a repellent for post-crossing axons (Bourikas et al., 2005). In contrast, Wnt signaling mediated by the graded expression of Wnt4 guides post-crossing commissural axons along the anteroposterior axis of the mouse spinal cord (Lyuksyutova et al., 2003, Wolf et al., 2008). These findings raise the question whether the two species use different morphogens for this navigational task or whether Shh and Wnts would cooperate (Stoeckli, 2006). In this study, we show that the role of Wnt ligands is conserved in the chicken spinal cord but with important mechanistic differences. In the chick, Wnt5a and Wnt7a rather than Wnt4 direct post-crossing axons rostrally upon floor-plate exit. But most importantly, in contrast to the mouse, where *Wnt4* was expressed in a gradient, expression levels of *Wnt5a* and *Wnt7a* did not change significantly along the anteroposterior axis of the chicken spinal cord. However, we demonstrate that a functional Wnt gradient is achieved by the graded distribution of the Wnt antagonist Sfrp1.

Our model is supported by several observations: first, expression analysis revealed that *Wnt5a* and *Wnt7a* transcripts are detected in and adjacent to the floor plate, respectively, and therefore fulfill the criteria for postcommissural axon guidance cues. Second, functional in vivo studies showed that downregulation of Wnt5a and Wnt7a, causes postcrossing guidance errors. Third, *Sfrp1* and *Sfrp2* are expressed within and adjacent to the floor plate, respectively, and display rostral^{low} to caudal^{high} gradients. Fourth, loss of Sfrp1 phenocopies the guidance defects seen after Wnt downregulation. Fifth, postcrossing commissural explants are attracted by Wnt5a and Wnt7a expressed by COS7 cells. Moreover, Wnt-

mediated attraction can be blocked by addition of purified Sfrp1, whereas Sfrp1 on its own had no effect on postcommissural axons in vitro. Sixth, overexpression of Wnt5a and Sfrp1 is sufficient to redirect postcommissural axons in vivo. Seventh, rostral versus caudal floor plate explants evoke different responses in postcrossing axons in vitro, whereas rostral floor plate has a significantly stronger activity. Importantly, the floor plate activity can be abolished by adding Sfrp1.

In the mouse, *Wnt4* was shown to be expressed in a gradient with high levels rostrally and low levels caudally, consistent with its attractive effect on post-crossing axons. In the chicken embryo downregulation of Wnt5a and Wnt7a, but not Wnt4, caused guidance errors in postcommissural axons. Two distinct but mutually not exclusive phenotypes were seen after loss of Wnt function: first, axons tend to stall at the contralateral border of the floor plate and second, caudal turns, a behavior never seen in control embryos, appeared. Similarly, Lyuksyutova et al. found that Wnt4 is growth promoting and can redirect axonal growth. Moreover, our in vivo overexpression experiments show that at least Wnt5a can redirect axons (Figure 2.7) and that Wnts stimulate growth in vitro (Figure 2.5). Several other studies support the idea that Wnt signaling can stimulate and direct the growth of axons at the same time (Lu et al., 2004; Li et al., 2009). This is in contrast to another morphogen, Shh, which attracts precommissural axons without affecting growth rate (Yam et al., 2009). Whether these different readouts, steering versus growth rate, are governed by distinct Wnt signaling pathways is not clear. A time course of experimental and control embryos revealed that the stalling phenotype was not a secondary effect or delay of axons due to manipulation of the embryos (not shown). The phenotypes seen in the chicken spinal cord after downregulation of Wnts were similar to those seen in the mouse (Lyuksyutova et al., 2003). However, the fact that commissural axon guidance was less severely disrupted in the chick might reflect the remaining activity of Shh (Bourikas et al., 2005).

Interestingly, neither *Wnt5A* nor *Wnt7A* was found to be expressed in a gradient along the anteroposterior axis of the spinal cord. In situ studies on spinal cord

cross-sections at different rostro-caudal levels and intensity measurements on open book preparations consistently showed that *Wnt* transcripts are not expressed in a longitudinal gradient. These findings seemed to contradict our functional results that indicated a role for Wnt5A and Wnt7A in directing post-crossing axons rostrally along the contralateral floor-plate border. However, an explanation for this paradox was found in the Sfrp family. Sfrps are known antagonists of Wnts (Jones and Jomary, 2002; Kawano and Kypta, 2003; Uren et al., 2000). Moreover, Sfrps were shown to interfere with the role of Wnts in commissural axon guidance (Lyuksyutova et al., 2003). Our finding that *Sfrp1* was expressed in a gradient in the floor plate with high concentrations caudally and low concentrations rostrally is consistent with a model that explains rostro-caudal pathfinding of post-crossing axons by an attractive Wnt activity gradient, formed by increasingly stronger inhibition of Wnts towards more caudal spinal cord levels. In contrast, in mouse the graded Wnt activity appears to be achieved by a transcriptional gradient, although the contribution of Sfrps cannot be ruled out and would be consistent with in vitro data (Lyuksyutova et al., 2003).
Our model is supported by in vivo analyses of Sfrp function. Downregulation of Sfrp1 phenocopied the axon guidance defects seen after removal of Wnt5a and Wnt7a. Furthermore, overexpression of *Sfrp1* rostrally, i.e. at thoracic levels, interfered with the rostral turn of post-crossing axons. Intriguingly, Sfrps seem to function differentially. Despite the fact that only Sfrp1 showed a clear phenotype in loss-of-function and gain-of-function studies, Sfrp2 and Sfrp3 would be appropriately expressed spatiotemporal (Figure 2.3). Similarly, Galli et al. showed that Wnt signaling is differentially inhibited by Sfrps. While Sfrp1 and Sfrp2 could block Wnt3a mediated signaling Sfrp3 could not (Galli et al., 2006). Differential antagonism could explain the weak or the absence of a phenotype after overexpression of Sfrp2 or Sfrp3, respectively (Figure 2.7).

The observations described above support a model where a Wnt activity gradient is shaped by a graded inhibition of Wnt rather than by the control of their transcription. They do not rule out the possibility of an additional direct effect of

Sfrp1 on post-crossing axons, however. Navigation of retinal ganglion cell axons was demonstrated to be directly influenced by Sfrp1 in both chick and frog (Rodriguez et al., 2005).

To further characterize Wnt and Sfrp function we performed in vitro studies on postcommissural explants. Indeed, the Wnt ligands had a strong impact on the growth of postcrossing commissural axons (Figure 2.5). These results, together with the in vivo overexpression of Wnts showing redirection of axons, explain the phenotype seen in in ovo RNAi. Removing Wnts, and therefore perturbing the gradient of Wnt activity, causes a loss of the directional cue (caudal turns) and a decrease in growth promotion (stalling at the floor plate border). Importantly, addition of Sfrp1 to the explant cultures had no effect on its own. However, Sfrp1 was potent in blocking the Wnt-mediated attraction of postcommissural axons. These results corroborate that ectopically expressed Sfrp1 in the in vivo situation does not directly act on postcommissural axons, but rather blocks endogenous Wnt function and thus indirectly redirects postcrossing axons.

Furthermore, experiments with precrossing explants showed that Wnts do not promote growth before axons have crossed, suggesting a temporally specific and excluding a more general effect (not shown). The determinant rendering precommissural axons insensitive and postcrossing axons sensitive to Wnts is still elusive. In 2008, however, Wolf and colleagues suggested that the p110 subunit of the PI3Kinase represents the switch between pre- and postcrossing behavior (Wolf et al., 2008). In vivo overexpression of p110 in mouse commissural axons caused them to turn randomly at the contra- or ipsilateral floor plate border. If this function is conserved between mouse and chick is not yet known.

The direct detection of a Wnt activity gradient in vivo is hampered by the fact that Wnt-mediated guidance of axons is unlikely conveyed by canonical signaling. Several lines of evidence point toward an involvement of β-catenin-independent signaling, rather than canonical Wnt pathway. Studies in the chicken hindbrain have linked Wnt5a and PCP signaling to motoneuron migration (Vivancos et al., 2009). Studies in mouse have ruled out the canonical pathway, because

anteroposterior guidance was normal in *Lrp6* knock-out mice (Lyuksyutova et al., 2003). Instead, the effect of Wnt4 on post-crossing axons required aPKC (Wolf et al., 2008). Additionally, transcription-independent signaling would be consistent with findings for Shh, where transcription was not required for its chemoattractive effect, despite the fact that signaling was still mediated by Smoothened (Yam et al., 2009). Studies in cerebellar granule cells revealed a divergent canonical Wnt pathway, independent of β-catenin, regulating growth cone morphology (Lucas et al., 1998; Ciani et al., 2004; Purro et al, 2008). Moreover, Wnt/Calcium signaling was implicated in steering cortical axons in vitro (Li et al., 2009). This excludes the use of helpful tools to detect Wnt activity, i.e. TCF/LEF reporter constructs and Axin2 expression (Barolo, 2006). In addition, a study using transgenic expression of β-Gal under the control of several consecutive TCF/LEF binding sites shows that putative (canonical) Wnt activity is exclusively found in the dorsal neural tube but not in the floor plate region where Wnt5a and Wnt7a are expressed (Maretti et al., 2003). We therefore chose another approach. If rostral lumbosacral floor plate has more Wnt activity (less Sfrp1) than caudal floor plate (more Sfrp1) the effect of floor plate explants on postcommissural explants should be different depending on the anteroposterior source of the floor plate. Indeed, rostral explants displayed a significantly stronger attraction on postcommissural explants than did caudal explants. This finding, however, could be explained by any other factors than Wnts present in the floor plate. To show that Wnts are mediating this effect we added purified Sfrp1. Indeed, the addition of Sfrp1 to the medium was sufficient to block part of the effect of floor plate tissue on axon growth suggesting that Wnts are the floor plate derived factors acting on commissural axons. The differential activity of floor plate depending on longitudinal origin and the possibility to block this activity by Sfrp1 clearly suggests the presence of a Wnt activity gradient.

In summary, based on our findings we propose a model for postcommissural axon guidance in the chicken embryo that implicates Wnts as directly acting attractive cues and Sfrps acting indirectly as regulators of Wnt activity. Chicken

commissural axons are therefore guided into the longitudinal axis by a simultaneous pushing and pulling of Shh (Bourikas et al., 2005) and Wnts (this study), respectively. Importantly, this study suggests for the first time a guidance mechanism which is not based on a transcriptional or diffusion gradient. Rather, a Wnt activity gradient, shaped by Sfrps, guides postcrossing commissural axons in the chicken spinal cord.

EXPERIMENTAL PROCEDURES

Preparation of in situ probes and dsRNA

Probes for in situ hybridization and dsRNA were produced from the following chicken ESTs: ChEST179l4 *(Wnt4*, bp 268 to 1177 of the ORF and 121 bp of 3'-UTR), ChEST2k9 (*Wnt5a*, bp 6 to 823 of the ORF), ChEST809e5 (*Wnt5b*), ChEST543m22 (*Wnt7a*, bp 421 to 1050), ChEST661e23 (*Wnt7b*), ChEST421c6 (*Wnt8a*), ChEST530d5 (*Wnt9b*), ChEST41h24 (*Wnt11*, 903 bp of 3' UTR), ChEST763j19 (*Sfrp2*, bp 195 to 867), and ChEST108h20 (*Sfrp3*, bp 495 to 1065 of the ORF and 465 bp of the 3'-UTR) (Geneservice Ltc, Cambridge, UK). Plasmids containing the ESTs were linearized with NotI and EcoRI (NEB). The *Sfrp1* plasmid (750 bp of the 3'-UTR) was linearized with BamHI and EcoRV (NEB). DIG-labeled probes were prepared and used for in situ hybridization as described previously (Mauti et al., 2006). DsRNA was generated by in vitro transcription as described previously (Pekarik et al., 2003). All sequences were carefully analyzed to avoid overlapping stretches that could lead to downregulation of family members. The specificity and the level of downregulation of the targeted gene were analyzed by in situ hybridization (Figure S3 and S5).

In ovo RNAi

Fertilized eggs (Hisex) were obtained from a local hatchery. The eggs were incubated at 38.5°C until the embryos reached the desired developmental stage (Hamburger and Hamilton, 1951). For functional analyses, plasmids or dsRNA for in ovo RNAi were injected and electroporated as detailed previously (Pekarik et al., 2003; Bourikas et al., 2005). In brief, HH18/19 embryos were injected into the central canal of the spinal cord with a solution containing 300 ng/µl dsRNA derived from the gene of interest and a plasmid encoding enhanced green fluorescent protein (EGFP) under the control of the β-actin promoter (50 ng/µl). Transfection of the floor plate or one half of the spinal cord was achieved by

electroporation with 5 pulses at 26V with a 1s interpulse interval (BTX Electro Square Porator ECM830; see Figure S2 and Bourikas et al., 2005). Our analysis of post-crossing commissural axon pathfinding was restricted to the lumbosacral level of the spinal cord. Only dye injection sites that were in the EGFP-positive areas of the spinal cord were considered for further analysis (see for example Figure S2). Embryos injected with the EGFP plasmid alone were used as controls and compared with untreated embryos.

Efficiency and specificity of target gene downregulation was verified in cryosections of the lumbosacral spinal cord of embryos at HH25/26. In the absence of specific antibodies for Wnts and Sfrps, we used in situ hybridization to measure downregulation of the target mRNAs (Mauti et al., 2007). For each condition at least 10 sections from 3 to 4 embryos were quantified using ImageJ software (Figures S3 and S5).

Analysis of post-crossing commissural axon pathfinding

The analysis of commissural axon trajectories in the lumbosacral spinal cord was performed as described previously (Bourikas et al., 2005; Perrin and Stoeckli, 2000; Stoeckli and Landmesser, 1995). Chicken embryos were sacrificed between HH25 and HH26 (Hamburger and Hamilton, 1951), the spinal cord was removed, opened at the roof plate (open-book preparation) and fixed for 30 min in 4% paraformaldehyde in PBS at room temperature. The lipophilic dye Fast-DiI (5 mg/ml in methanol; Molecular Probes) was applied to the cell bodies of dorsolateral commissural neurons. To allow for diffusion of the dye, the open-book preparations were kept in PBS at 4°C for 2 to 3 days. The spinal cords were mounted in PBS between two coverslips sealed with high vacuum grease (Dow Corning). The phenotypes were quantified as normal (axons turn rostrally along the contralateral floor-plate border and no more than 50% of the axons stall within the floor plate), and strong (commissural axons found to turn caudally or more than 50% of the axons stalling before reaching the contralateral floor-plate border). Caudal turns were never observed when only dorsolateral commissural axons were analyzed as done here and in our previous studies (Bourikas et al.,

2005). On average 7 injection sites per spinal cord were analyzed. Only embryos with more than three injection sites were included in the analysis. Furthermore, only injection sites that were exclusively in the area of the dorsolateral border cells were analyzed. Injection sites that were too ventral were not considered as this may have labeled more ventrally located populations of commissural neurons with divergent axonal trajectories.

Ectopic expression of *Wnts* and *Sfrps*

For gain-of-function experiments, the open reading frames of chicken *Wnt5a* and *Wnt7a* were cloned in the pMES vector (kindly provided by C. Krull). Total RNA was purified from spinal cords of HH25/26 chicken embryos using TRIzol (Invitrogen) according to the manufacturer's protocol and used as template for cDNA synthesis with the SuperScript Choice System (Invitrogen) and the T7-(T)24 primer: 5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGC GG(dT)24-3'. Specific primers for *Wnt5a* were 5'-CTAGTCTAGAA TGGAGAAATCCACTGCAGTATTAA-3' (forward) and 5'-CGGAATTCC TATTTGCACACAAACTGGTCC-3' (reverse). For *Wnt7a* cloning, the forward primer was 5'-CTAGTCTAGAATGAACAGGAAAACAAGGC-3' and the reverse primer was 5'-CGGAATTCTCACTTACAGGTATATACTTCTGTT-3'. XbaI and EcoRI restriction sites were introduced to the forward and reverse primers for cloning the PCR fragments into the pMES vector. The pMES plasmid contains an IRES sequence followed by *EGFP*, thus allowing for direct detection of transfected cells. The pCIG-*Sfrp1-myc/his*-IRES-*EGFP* plasmid was generated from the pCDNA3.1-*Sfrp1-myc/his* expression vector (Esteve et al., 2003). pCIG-*Sfrp2-myc/his* and pCIG-*Sfrp3-myc/his* plasmids were kindly provided by Laura Burrus (Galli et al., 2006). In the pCIG plasmid, the IRES sequence is followed by *EGFP* containing a nuclear localization sequence (NLS). The pMES-*Shh* was described previously (Bourikas et al., 2005). For overexpression we injected 500 ng/µl of the plasmid. When the plasmid did not contain an IRES sequence followed by EGFP, we coinjected 50 ng/µl of a plasmid encoding EGFP under the control of the β-actin promoter to visualize transfected cells. To reverse the

functional gradient of endogenous Wnt5a and Wnt7a, the respective plasmids were injected into the central canal of the embryonic spinal cord and electrodes were positioned at caudal levels of the lumbosacral spinal cord. For the ectopic expression of Sfrps the electrodes were positioned at thoracic levels.

In vitro assays

For cultures of post-crossing commissural axons, spinal cords were dissected at HH25 as described previously (Bourikas et al., 2005). Explants were cultured in collagen gels alone or with COS7 cells that were transfected with lipofectamine 2000 (Invitrogen) either with an empty vector (pcDNA3.1; Invitrogen) or with pcDNA3.1 containing the cDNA of *Wnt5a* or *Wnt7a*. After 20-24h cells were fixed in 4% PFA for 1h. Axons were visualized by exposure to 2U/ml Oregon Green phalloidin for 20 min. The explants were analyzed with ImageJ as described by Wolf and colleagues (Wolf et al., 2008). Axon growth of experimental explants was normalized to control explants (w/o cells) for each experiment. Values from three to five experiments were pooled and p values calculated with the Student's t-test (paired and two-tailed distributions). Wnt activity was blocked by adding recombinant human Sfrp1 (R&D Systems) to a final concentration of 1µg/ml.

To demonstrate the graded activity of Wnts along the anteroposterior axis rostral and caudal floor-plate explants were cultured together with post-crossing commissural axons as depicted in Figure 2.6A. One open-book preparation from a HH25 lumbosacral spinal cord was dissected into 3 explants containing commissural neurons and 2 floor-plate pieces (one rostral and one caudal to the commissural explants, R and Ca, respectively). The commissural neuron explants were randomly assigned to the three possible configurations a, b, and c, cultured for 20 to 24h, stained and analyzed as described above.

Acknowledgements

We thank S. Arber, L. Burrus, A. Klar, C. Krull, and M. Tessier-Lavigne for reagents and members of the lab for critically reading the manuscript. This work was supported by grants of the Swiss National Science Foundation and the NCCR Brain Plasticity and Repair.

Supplementary figures

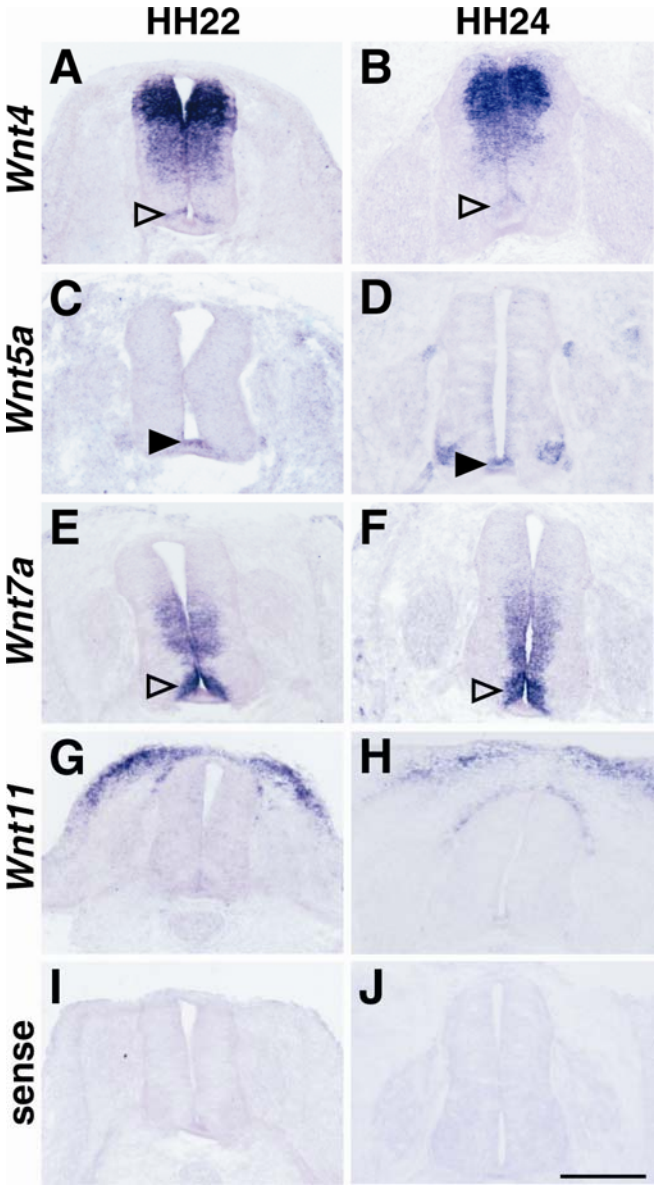


Figure S1 Analysis of *Wnt* expression in the lumbosacral spinal cord of the chicken embryo by in situ hybridization.
In contrast to mouse, *Wnt4* is not expressed in the floor plate but at high levels in the dorsal spinal cord in chicken embryos at HH22 (A) and HH24 (B). There is a small ventral expression domain adjacent to the floor plate (open arrowhead in A,B). At HH22, the time when axons of dorsolateral commissural neurons have reached the floor plate and cross the midline and at HH24, when they turn into the longitudinal axis, both *Wnt5a* (C,D) and *Wnt7a* (E,F) are expressed in a pattern that is compatible with a role as post-crossing commissural axon guidance cue. *Wnt5a* is expressed in the floor plate (arrowhead in C,D), whereas *Wnt7a* is found in the domain adjacent to the floor plate (arrowhead in E,F), where axons exit and turn into the longitudinal axis. In addition, *Wnt7a* was also expressed in the ventral ventricular zone of the spinal cord at both stages. *Wnt11* is not expressed in the spinal cord (G,H) and was used as a negative control. The sense probes did not result in any staining, as indicated for example for the sections processed with the *Wnt5a* sense probe (I and J). Bar: 200 µm.

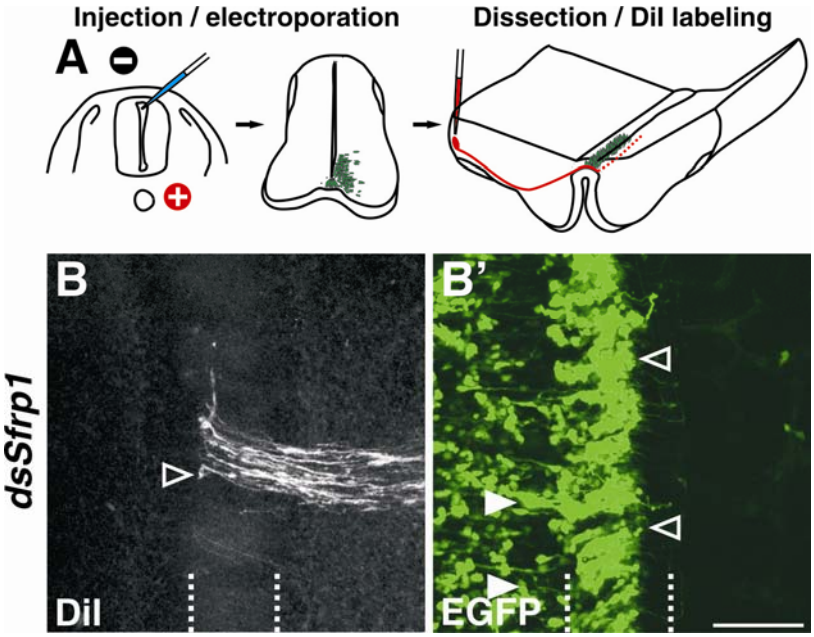


Figure S2 Gene silencing by in ovo RNAi.
For the functional analysis of Wnts and Sfrps, we used in ovo RNAi, a method that we have established for specific gene silencing in chicken embryos (Pekarik et al., 2003; Bourikas et al., 2005; Mauti et al., 2007). For gene silencing a fragment of the open reading frame or the untranslated 5' or 3' sequence was selected by BLAST to avoid fragments that contain overlapping sequences with non-target genes. Nucleic acids were efficiently transfected into spinal cord cells by in ovo electroporation. For this study electrodes were positioned as shown in (A) to target the floor plate and the immediately adjacent area. Two days after electroporation embryos were sacrificed and axons were traced by DiI as detailed in the Experimental Procedures (see also Perrin and Stoeckli, 2000). An example for silencing *Sfrp1* is shown (B and B'). For the analysis of post-crossing commissural pathfinding we only considered injection sites that were in the area of EGFP expression. Injection sites at the borders of the EGFP-positive areas were not included. Bar: 60 µm.

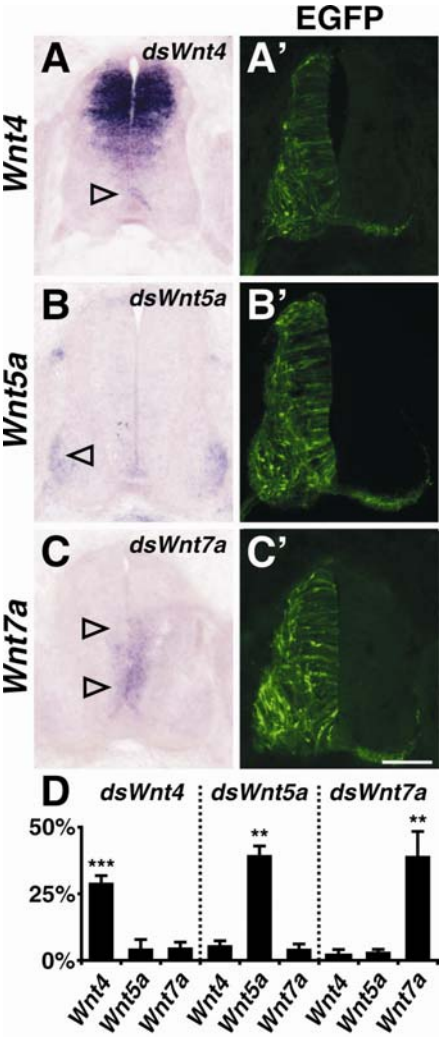


Figure S3 In ovo RNAi specifically downregulates the targeted *Wnt* gene.
In the absence of specific antibodies for Wnts we used in situ hybridization to demonstrate specificity and efficiency of target gene silencing by in ovo RNAi as detailed previously (Mauti et al., 2007). Transverse sections of embryos were hybridized with probes derived from *Wnt4* (A), *Wnt5a* (B), or *Wnt7a* (C). Transfection efficiency was verified by a co-electroporated plasmid encoding EGFP (A',B',C'). In an embryo treated with dsRNA derived from *Wnt4* (A), *Wnt4* mRNA levels were reduced by 29.2±1.5% in the area that was analyzed from the electroporated side compared to the equivalent area from the non-electroporated side (open arrowhead in A; D). No changes in the expression of *Wnt5a* and *Wnt7a* were detected (D). Similarly, targeting *Wnt5a* (B) specifically reduced *Wnt5a* mRNA in the electroporated area (open arrowhead in B) by 35.7±1.9% without affecting *Wnt4* and *Wnt7a* (D). *Wnt5a* mRNA levels were measured in motoneurons of the lumbosacral spinal cord rather than the floor plate in order to facilitate quantification. Targeting Wnt7a (C) reduced *Wnt7a* mRNA levels in the electroporated area (open arrowheads in C) by 39.3±5.2% without affecting *Wnt4* and *Wnt5a* levels (D). Bar: 100 µm.
Values are given ±SEM (standard error of the mean). The comparison of the differences in expression levels between the electroporated and the control side was highly significant. P values were 0.00053 and 0.00021 for *Wnt4* compared to *Wnt5a* and *Wnt7a*, respectively. P values for the comparison between *Wnt5a* as target with non-targeted *Wnt4* was 0.00419 and 0.00375 for *Wnt7a*. Targeting *Wnt7a* reduced only *Wnt7a* with P values of 0.00228 (*Wnt4*) and 0.00241 (*Wnt5a*). For each condition 3 embryos with an average of 8 sections (range 5-11) were analyzed.

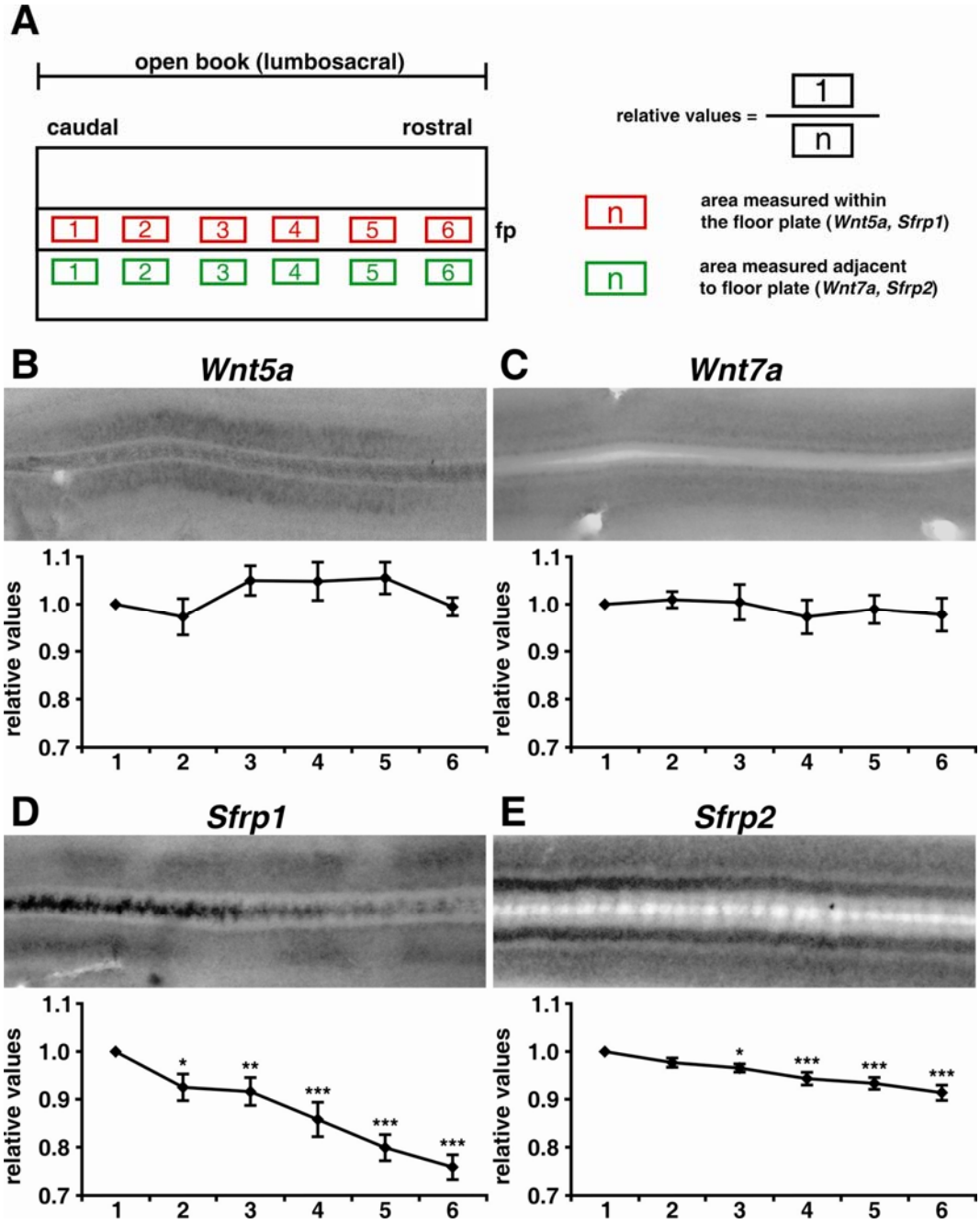


Figure S4 *Sfrp1* and *Sfrp2*, but neither *Wnt5a* nor *Wnt7a* are expressed in a gradient along the rostro-caudal axis of the embryonic chicken spinal cord.
Open-book preparations of spinal cords were used for in situ hybridization with probes for *Wnt5a*, *Wnt7a*, *Sfrp1*, and *Sfrp2*. For quantification of the expression levels relative pixel intensities in 6 defined areas along the rostro-caudal axis were measured as indicated in (A). The most caudal value [1] was set to 1.0 and all more rostral positions [2-6] were normalized to value [1]. The number of spinal cords included in the analysis was 10 for Wnt5a (B), 6 for Wnt7a (C), 11 for Sfrp1 (D), and 12 for Sfrp2 (E). Gradients were only found for *Sfrp1* and *Sfrp2*, where measurements for positions [2] – [6] compared to position [1] were significantly different (pairwise Student t-test). Asterisk indicates $p<0.05$, two asterisks indicate $p<0.01$, three asterisks indicate $p<0.001$ for pairwise comparison of indicated position relative to value at position [1].

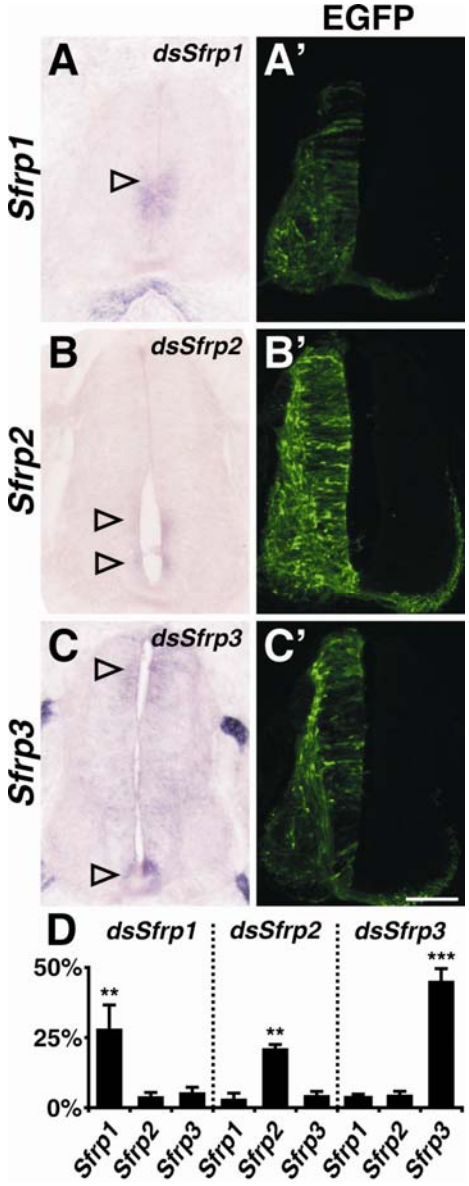


Figure S5 In ovo RNAi specifically downregulates the targeted *Sfrp* gene.
In the absence of specific antibodies for Sfrps we used in situ hybridization to demonstrate specificity and efficiency of target gene silencing, as shown for *Wnts* (Figure S3). Transverse sections of embryos were hybridized with probes derived from *Sfrp1* (A), *Sfrp2* (C), or *Sfrp3* (E). Transfection efficiency was verified by a co-electroporated plasmid encoding EGFP (B,D,F). In an embryo treated with dsRNA derived from *Sfrp1* (A,B), *Sfrp1* mRNA levels were reduced by 27.9±3.7% when an area from the electroporated (open arrowhead in A) was compared to the equivalent area from the non-electroporated side (G). No changes in the expression of *Sfrp2* and *Sfrp3* were detected (G). Similarly, targeting *Sfrp2* specifically reduced *Sfrp2* mRNA in the electroporated area (open arrowhead in C) by 20.9±0.7% without affecting the other *Sfrps* (G). *Sfrp3* mRNA levels were reduced by 44.9±2.5% after electroporation of *Sfrp3* dsRNA (open arrowhead in E) without an effect on *Sfrp1* and *Sfrp2* (G). P values were 0.00022 (*Sfrp1* versus *Sfrp2*) and 0.00413 (*Sfrp1* versus *Sfrp3*) when *Sfrp1* was targeted, 0.00528 (*Sfrp2* versus *Sfrp1*) and 0.00605 (*Sfrp2* versus *Sfrp3*) when *Sfrp2* was targeted, and 0.00008 (*Sfrp3* versus *Sfrp1*) and 0.00009 (*Sfrp3* versus *Sfrp2*) when *Sfrp3* was targeted. Values are given ±SEM. For each condition at least 3 embryos with an average of 10 sections (range 8-13) were analyzed. Bar: 100 µm.

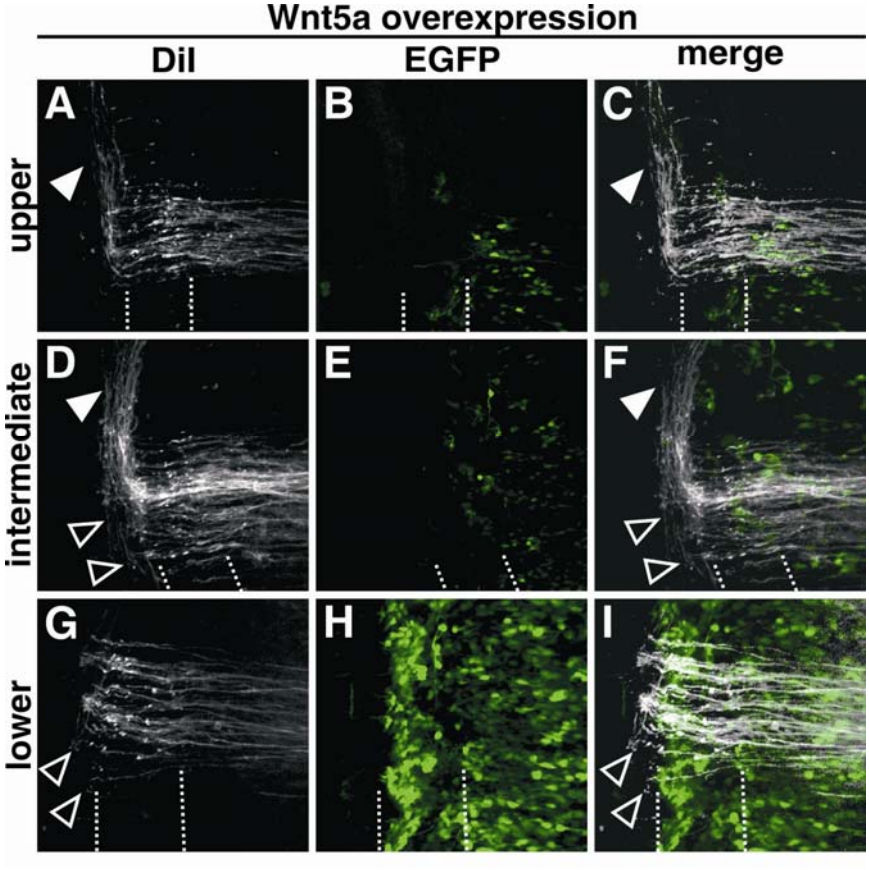


Figure S6A-I Perturbation of graded Wnt activity resulted in the expected pathfinding errors of postcommissural axons.
Overexpression of *Wnt5a* in the caudal spinal cord reversed levels of active Wnt (A-I). Expression of ectopic *Wnt5a* was monitored by the expression levels of EGFP (B,E,H). At upper lumbosacral levels very little or no ectopic expression of *Wnt5a* was found in the floor plate (B). As expected post-crossing commissural axons at this level did not show any changes in their pathfinding behavior (A and C for a merged image) compared to control embryos (not shown). More caudally, at intermediate levels, ectopic *Wnt5a* expression levels were slightly higher resulting in pathfinding errors of some postcommissural axons (open arrowheads in D). The majority of the axons still correctly turned rostrally along the contralateral floor-plate border (arrowhead in D, merged image in F). At caudal lumbosacral levels with high levels of ectopic *Wnt5a* (H) pathfinding of post-crossing axons was severely affected (G; I merged image). Most axons failed to turn, only very few axons turned caudally most likely due to high Shh levels in the caudal floor plate (open arrowheads).

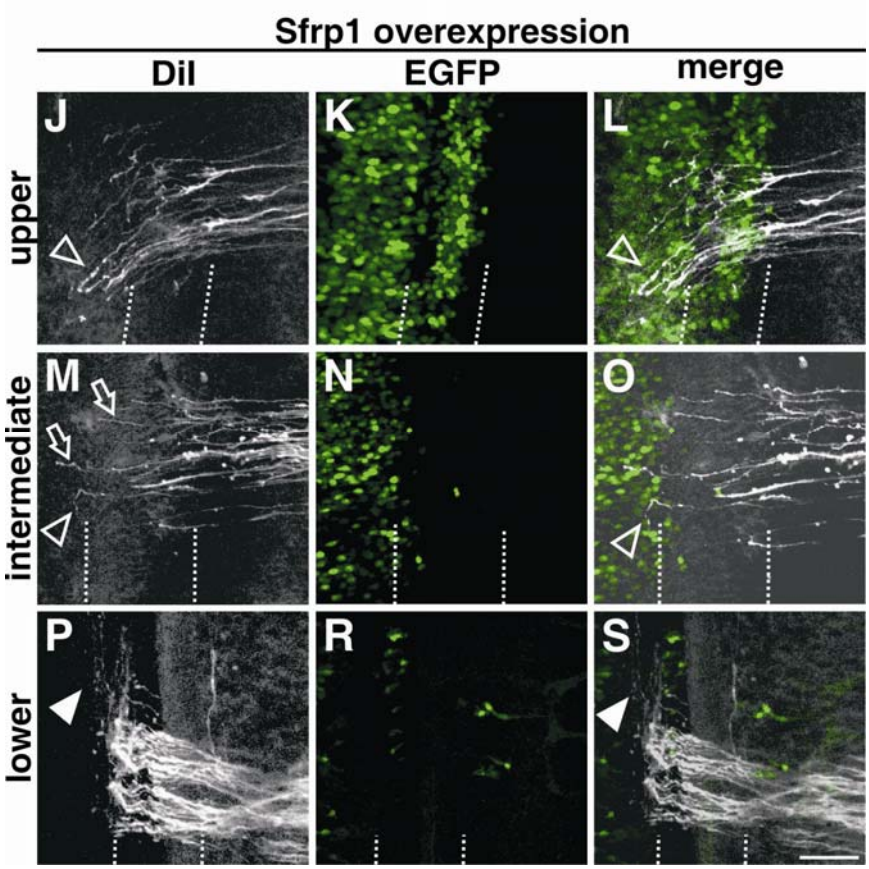


Figure S6J-S Perturbation of graded Wnt activity resulted in the expected pathfinding errors of postcommissural axons.
Similarly, the gradient of active Wnt was perturbed by ectopic expression of Sfrp1 at rostral levels (J-S). High levels of *Sfrp1* at upper lumbosacral levels (K, L for merged image) inhibited Wnt excessively, resulting in a reduced attraction of post-crossing axons in rostral direction (arrowhead in J). At intermediate levels (M-O), axonal behavior was randomized. Axons mostly failed to turn in any direction (open arrowhead in M and O). At caudal-most levels (P-S) with low levels of ectopic (R) but high levels of endogenous *Sfrp1* axon pathfinding was not different from control embryos (arrowheads in P,S).

4. Annex

Additional material and methods

Analysis of neural tube patterning

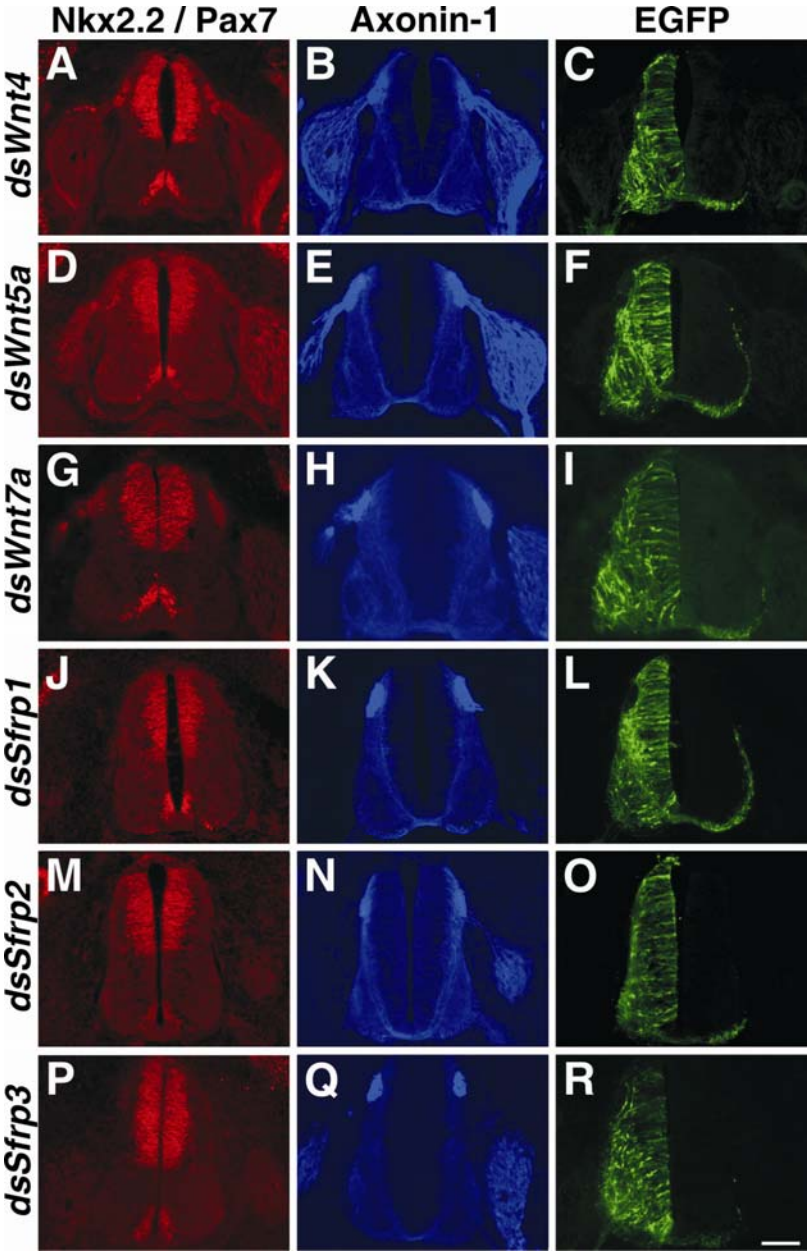
For the analysis of neural tube patterning, we used 25 µm-thick cryosections of the lumbosacral spinal cord of non-treated and experimental embryos. Immunostaining was done as described previously (Perrin et al., 2001). The induction of ventral and dorsal cell types was assessed by Nkx2.2 (74.5A5) and Pax7 staining, respectively. Monoclonal antibodies (obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA) were mixed for staining of transverse spinal cord sections after downregulation or overexpression of Sfrps and Wnts. Antibodies against Axonin-1 were used to stain precommissural axons. The floor plate structure was monitored by Shh (5E1) and HNF3β (4C7). Fluorescent secondary antibodies (goat anti-mouse IgG-Cy3 (Jackson ImmunoResearch Newmarket, Suffolk, UK) and goat anti-rabbit Alexa350 (Molecular Probes)) were used at a dilution of 1:250.

In vitro assays

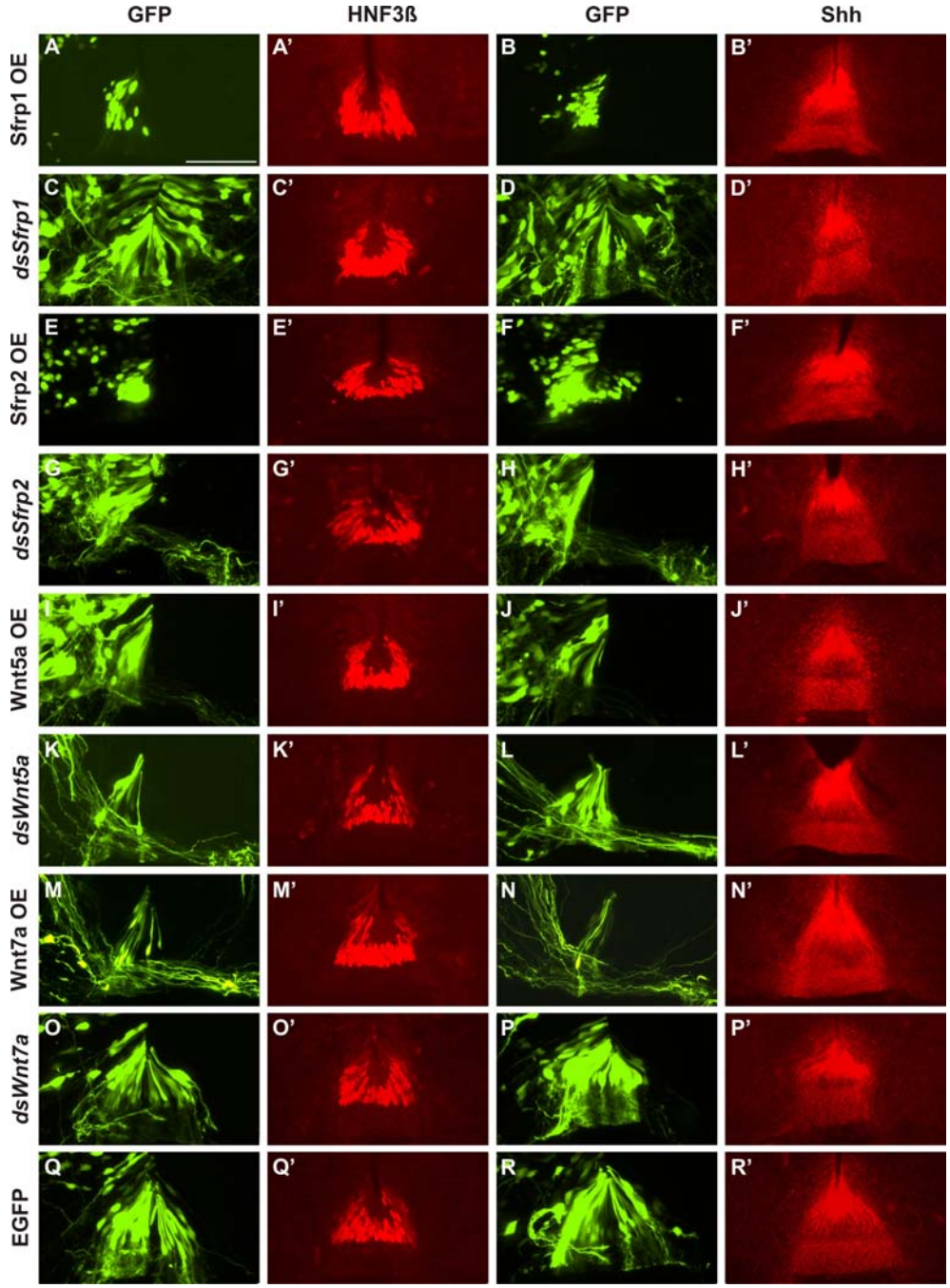
To demonstrate that axons extending from HH25 explants are indeed post-crossing commissural axons, we used a construct for the expression of MARCKS-GFP (kindly provided by S. Arber) under the control of the Math1-promoter that is expressed specifically in dorsal commissural axons. Chicken embryos were electroporated at HH18 with Math1-MARCKS-GFP (1µg/µl) and postcrossing explants dissected at HH25. GFP fluorescence was detected without staining after 24 h of incubation.

For cultures of pre-crossing commissural axons, chicken spinal cords were dissected at HH22/23 and explants of dorsal spinal cord (without floor plate) were cultured for 20-24 h alone or with either mock-transfected or Wnt-expressing COS7 cells. As positive control HEK293T cells stably expressing Netrin-1 (kindly provided by Dr. M. Tessier-Lavigne) were used (Shirasaki et al., 1996).

Additional figures

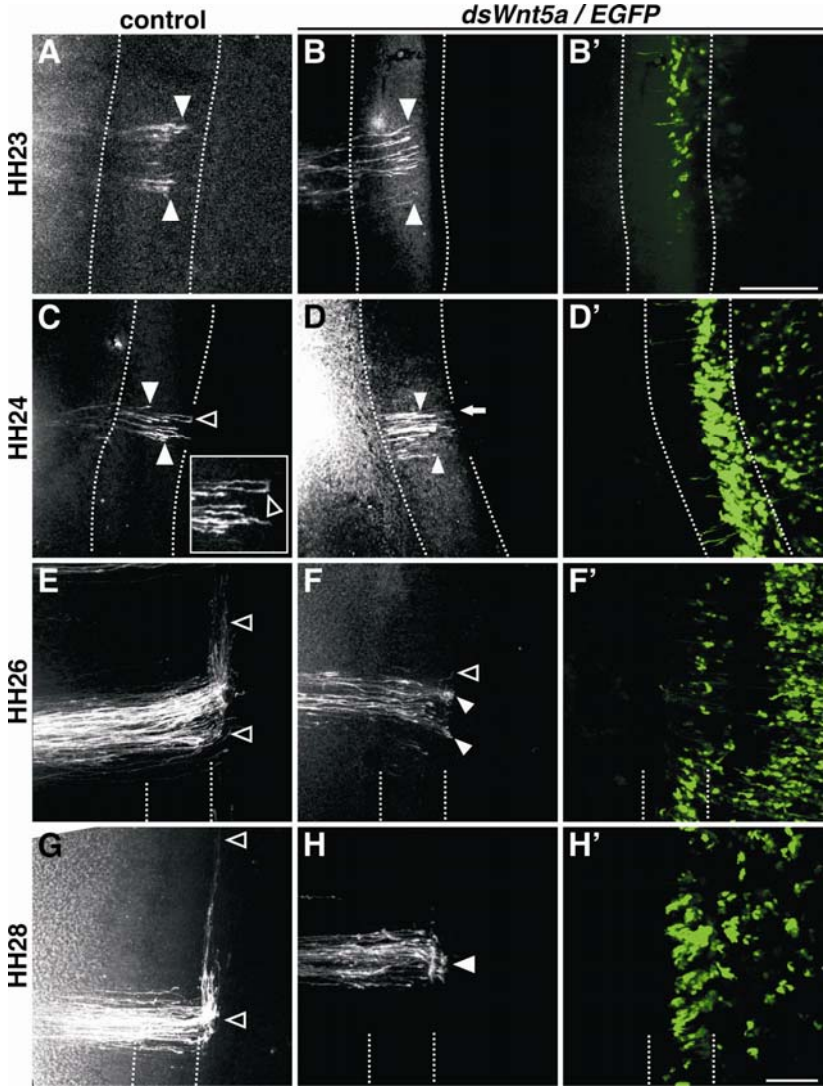


Annex1 Downregulation of *Wnts* and *Sfrps* during commissural axon pathfinding does not affect neural tube patterning.
After silencing the target gene by in ovo RNAi embryos were sacrificed at HH25. Spinal cord patterning and growth of commissural axons were analyzed on cryosections of the lumbosacral spinal cord. Patterning was assessed by a combined staining for Nkx2.2 and Pax7 (A,D,G,J,M,P). Commissural axons were stained with an anti-Axonin-1 antibody (B,E,H,K,N,Q). Transfection efficiency was controlled by co-electroporation of a plasmid encoding EGFP (C,F,I,L,O,R). None of the experimental embryos showed any difference when compared to non-injected control embryos (not shown). Bar: 100 µm.



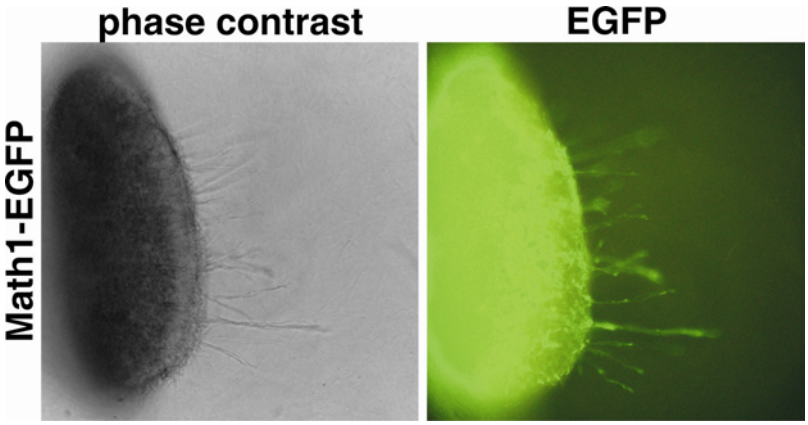
Annex2 Floor plate markers Shh and HNF3β are unaffected after in ovo RNAi and overexpression of Wnts and Sfrps.

To rule out unspecific effects due to changes in floor plate morphology or the expression of other floor plate-derived guidance cues we checked the protein levels of the floor plate marker HNF3β and the axon guidance cue Shh. The area of transfection is indicated by the expression of GFP (A,C,E,G,I,K,M,O,Q and B,D,F,H,J,L,N,P,R) for all conditions. The expression of HNF3β was not effected compared to EGFP control (Q') after in ovo RNAi or overexpression of Wnts and Sfrps (A',C',E',G',I',K',M',O'). More importantly, the expression of the commissural axon guidance cue Shh was also unaffected (B',D',F',H',J',L',N',P') compared to EGFP control (R').

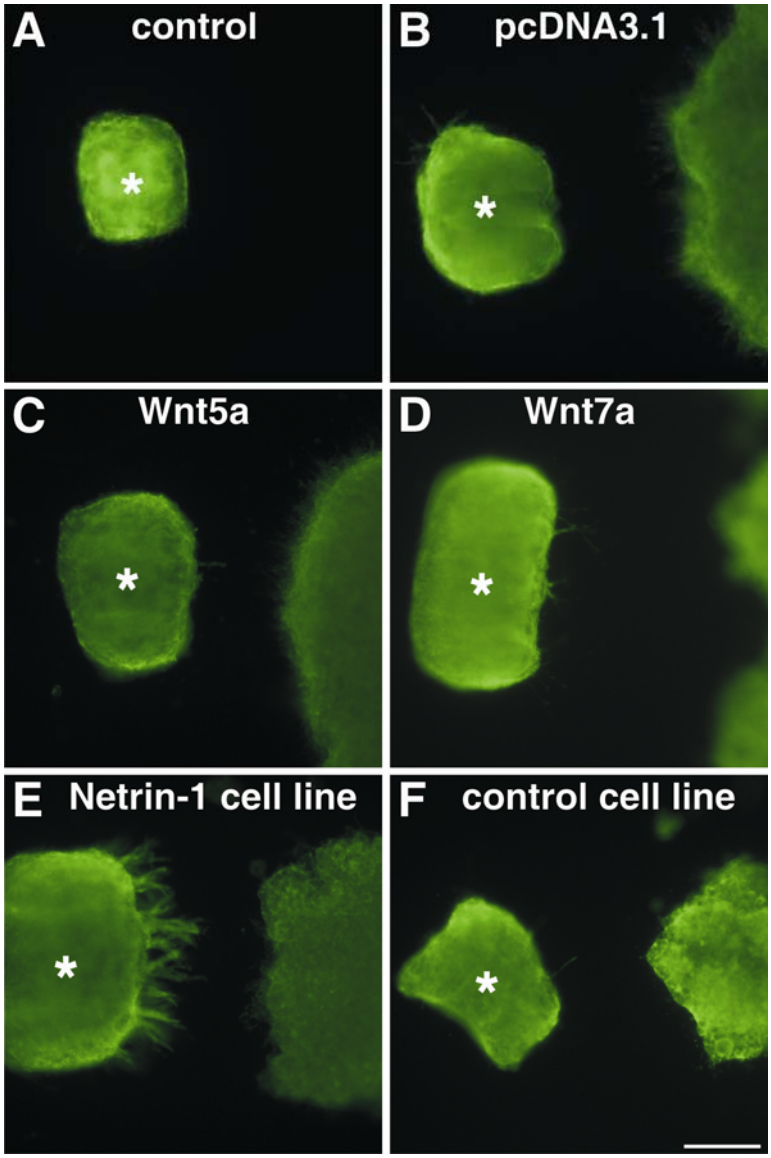


Annex3 Downregulation of Wnt5a by in ovo RNAi perturbs guidance but does not interfere with growth of commissural axons.

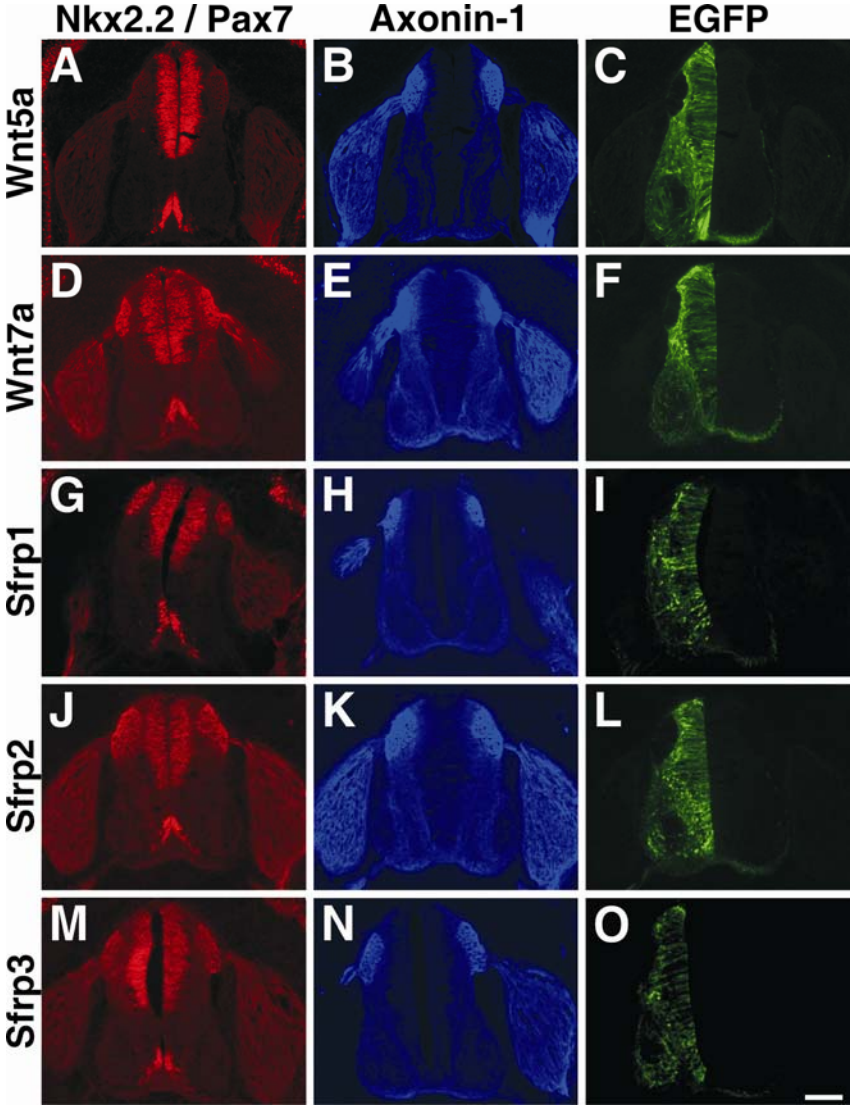
To rule out an effect on growth rate rather than guidance of commissural axons, we silenced Wnt5a by in ovo RNAi at HH18 but sacrificed embryos at different time points. Commissural axons were labeled with DiI to compare their growth pattern between untreated control (A,C,E,G) and experimental embryos (B,D,F,H). At HH23 commissural axons are crossing the floor plate in both control embryos and embryos lacking Wnt5a (arrowheads in A and B, respectively). A few hours later, at HH24, axons reach the contralateral border of the floor plate and turn into the longitudinal axis in control embryos (open arrowhead in C; higher magnification in insert). Some axons in embryos lacking Wnt5a have reached the contralateral floor-plate border but no turns were found at HH24 (arrow in D). At HH26 axons had turned and extended along the longitudinal axis for a considerable distance in non-treated embryos (open arrowheads in E). In experimental embryos lacking Wnt5a axons were still at the floor-plate exit site and turned randomly rostrally or caudally but mostly failed to extend along the longitudinal axis for more than a very short distance at HH26 (arrowheads in F indicate axons turning caudally, open arrowhead indicates axons turning rostrally). Even when sacrificed one day later at HH28 axons still lingered at the floor-plate exit site in embryos lacking Wnt5a (arrowhead in H). In contrast, axons in untreated control embryos had extended further along the contralateral floor-plate border (arrowheads in G). EGFP expression was used to confirm efficient targeting of nucleic acids into the floor-plate area (B',D',F',H'). Bar: 60 µm.



Annex4 Axons originating from explants are post-crossing commissural axons.
Membrane-bound GFP was specifically expressed in dorsal commissural neurons under the control of the Math1 promoter (Zisman et al., 2007). The comparison between phase contrast images and images taken with the appropriate filter setting to visualize GFP demonstrated that axons leaving the explant were indeed Math1-GFP-positive dorsal commissural axons. Bar: 200 µm.



Annex5 Pre-crossing commissural axons are not sensitive to Wnts.
Embryos were dissected at HH23 or earlier to obtain explants of pre-crossing commissural neurons. No neurites extended from these explants when they were cultured in 3D collagen cultures (A) or together with mock-transfected COS cells (B). Neither Wnt5a- (C), nor Wnt7a-expressing (D) COS cells promoted neurite growth from pre-commissural axons. As a positive control, pre-commissural explants were co-cultured with HEK cells expressing Netrin-1 (E). Control HEK cells did not promote growth of pre-commissural axons (F). Bar: 200 µm.



Annex6 Overexpression of Wnts and Sfrps during the time window of commissural axon guidance does not change the patterning of the neural tube.
Overexpression of *Wnts* (A-F) or *Sfrps* (G-O) at HH18 did not induce changes in Nkx2.2 or Pax7 expression (A,D,G,J,M), indicating that the patterning of the neural tube was not different from untreated control embryos (not shown). Similarly, Axonin-1 staining (B,E,H,K,N) revealed no difference in commissural axon growth toward the floor plate in embryos overexpressing *Wnt5a* (B), *Wnt7a* (E), *Sfrp1* (H), *Sfrp2* (K), or *Sfrp3* (N) compared to non-treated control embryos (not shown). EGFP expression from a co-electroporated plasmid was used as a transfection control (C,F,I,L,O). Bar: 100 µm.

5. Outlook

In my thesis I could demonstrate that the role of Wnt proteins in postcrossing commissural axon guidance is conserved in the chicken embryo. Importantly, I was able to show that Wnts do not act alone, but that in vivo modulation of Wnt signaling activation is an important factor in axon guidance. This function was covered by secreted frizzled-related proteins (Sfrps), known Wnt antagonists. Further research should concentrate on the following questions: What is the Wnt pathway that regulates commissural axon guidance? How do commissural axons switch from a precrossing Wnt-insensitive to a postcrossing Wnt-sensitive state? Do Wnts interact with other guidance cues?

What is the Wnt pathway that regulates commissural axon guidance? Little is known about the downstream Wnt signaling cascade regulating cytoskeleton dynamics to steer postcrossing commissural growth cones. Wnt/Calcium and Wnt/PCP pathways share similarities to known intracellular guidance mechanisms (see Wnt chapter). Calcium signaling has already been established as a mechanism mediating growth cone responses (Zheng and Poo, 2007). On the other hand, Wnt/PCP pathway regulates cytoskeleton acting through small GTPases of the Rho family (Schlessinger et al., 2009). Similarly, classical axon guidance cues signal via GAPs and GEFs to regulate Rho family GTPases and subsequently the cytoskeleton (Dickson, 2002). Cerebellar granule cell axons revealed a divergent canonical Wnt pathway modulating growth cone morphology (Salinas, 2007). Additionally, the Par complex (a protein complex consisting of Par3, Par6, aPKC, and Cdc42 regulating apico-basal polarity formation in epithelia), was suggested to govern Wnt-mediated commissural axon guidance in rodents (Wolf et al., 2008). It is likely that one of these noncanonical Wnt pathways regulates the longitudinal guidance of commissural growth cones; possibly in concert with the Par complex. Dissection of signaling pathways in axon guidance is not a simple task. With in ovo RNAi in hand, however, we have the possibility to modulate different signaling components

efficiently and specifically in space and time. So, signaling genes could be downregulated specifically in dI1 neurons at the time when their axons start to grow into the longitudinal axis. This would prevent interference with earlier signaling functions which are likely for all Wnt signaling components (Alvarez-Medina et al., 2007; Lei et al., 2006; Megason and McMahon, 2002). Another contact surface to gain further insight into downstream Wnt signaling in axon guidance is the fact that recruitment of different co-receptors leads to the activation of distinct signaling cascades (Schweizer and Varmus, 2003; Yamamoto et al., 2008). Thus the presence and absence of co-receptors and co-factors, i.e. Lrp5/6, Ryk, Ror2, and Cthrc1, can narrow down candidate signaling pathways.

How do commissural axons switch from a Wnt-insensitive to a Wnt-sensitive state? Precrossing mouse and chick axons are not sensitive to Wnt ligands, but become sensitive after crossing the midline (Lyuksyutova et al., 2003; this thesis). Virtually nothing is known about this change in sensitivity. Preliminary results presented by Wolf and colleagues revealed a possible switch between the Wnt-insensitive pre- and the Wnt-sensitive postcommissural axons. In vivo overexpression of a kinase deficient p110 subunit of PI3K resulted in caudal turning of commissural axons after crossing. Surprisingly, overexpression of the wildtype version of p110 caused ipsilateral turning of commissural neurons. Wolf and colleagues suggest that p110 subunits are only expressed during turning. It is not clear yet how the spatiotemporal distribution of p110 is regulated. Two main mechanisms are possible: either the expression of the gene is only initiated during/after crossing or the protein is only localized to the growth cone membrane after crossing. Both mechanisms were observed for different axon guidance cues in chicken commissural axons. First, Shh acts as a repulsive cue for postcrossing axons. This effect is due to the transient expression of Hedgehog-interacting protein (Hhip) at the stage when commissural axons reach the contralateral side of the midline (Bourikas et al., 2005). Second, Slits only mediate repulsion after axons have crossed (Zou et al., 2000). Work in our group

suggests that Robo1 is present in precrossing growth cone vesicles but is only shuttled to the membrane after midline crossing (Philips et al., submitted). The Wnt insensitivity of precrossing could therefore be due to a change in gene expression (of a receptor like Hhip or an intracellular modulator like PI3K). Otherwise, it could be a change of growth cone sensitivity due to temporally regulated protein insertion. These complex questions could be nicely addressed with the chicken embryo. The functional read-out of in ovo RNAi allows discriminating between pre- and postcrossing phenotypes. Furthermore, in vitro assay with pre- and postcrossing explants, as used in this thesis, could nicely reveal the Wnt sensitivity of commissural axons depending on the presence, i.e. after overexpression of p110, or absence, i.e. after in ovo RNAi, of candidate genes.

Do Wnts interact with other guidance cues? Another interesting aspect of commissural axon guidance in the chick is the possible cooperation of the two guidance cues Shh and Wnt (Stoeckli, 2006). It is not clear whether these two morphogens act independently or are connected somehow. Presomitic mesoderm for instance is patterned by the antagonistic action of Wnt and Shh. Shh antagonizes Wnt activity via the upregulation of Wnt inhibitor Sfrp2 (Lee et al., 2000). Reminiscent of the molecular mechanisms in presomitic patterning Shh and Sfrps are expressed in similar gradients in the chicken floor plate (Bourikas et al., 2005; this thesis). Moreover, Wnt activity in axonal steering is modulated by Sfrp1 (this thesis). Another interesting twist to the idea that Shh and Wnts interact in axon guidance was given by a recent study elucidating the neurogenic potential of the ventral midline. Floor plate tissue has different properties along the neuraxis depending on the presence or absence of Shh. This might, as Joksimovic and colleagues suggest, be governed by antagonistic actions of the canonical Wnt pathway and Shh. They demonstrated that β-catenin signaling was sufficient and necessary to antagonize Shh expression (Joksimovic et al., 2009). It appears possible therefore that Shh antagonizes Wnt (Lee et al., 2000) and vice versa (Joksimovic et al., 2009). So, to fully understand

the mechanism of Wnt function in axon guidance, one needs to see beyond one's own nose and elucidate possible interactions with other guidance systems.

Wnt signaling is involved in various human diseases (Clevers, 2006; MacDonald et al., 2009). The importance of Wnts is for instance well known in the field of cancer biology, not least because Wnt1 was initially identified as an oncogene, and also in stem cell research. But Wnt signaling is also affecting the nervous system. The deficits seen due to aberrant Wnt signaling range from defects in neural tube closure to Alzheimer's disease (De Ferrari et al., 2007; Kibar et al., 2007). The molecular mechanisms are however often poorly understood, reflecting the importance of further basic research in this field. Moreover, molecular mechanisms acting during nervous system development can turn out to be important for similar functions in the adult, as is illustrated by Wnt-Ryk signaling. Wnt-Ryk signaling was found to act as a repulsive guidance system steering corticospinal tract (CST) axons during development (Liu et al., 2005). CST axons were repelled by decreasing gradients of Wnt proteins. This effect was mediated by the receptor tyrosine kinase Ryk. A recent study suggests that Wnt-Ryk signaling could be important in axon regeneration after spinal cord lesions (Liu et al., 2008). In there study, Liu and colleagues showed that Wnt signaling components are upregulate in the lesioned spinal cord and inhibit axonal sprouting. Thus, mechanisms revealed in developmental neuroscience can help us understand the function of the adult brain and its regenerative properties. Unraveling the molecular mechanisms of Wnt signaling in neural development is therefore of great therapeutic importance.

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	ZNZ Symposium, Zürich, CH
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	ZNZ Symposium, Zürich, CH
2009	Wnt Meeting, Arolla, CH

List of publications

Andrin Wacker*, Elena Domanitskaya*, Olivier Mauti, Thomas Baeriswyl, Pilar Esteve,Paola Bovolenta, and Esther T. Stoeckli. Sonic hedgehog guides post-crossing commissural axons both directly and indirectly by regulating Wnt activity (submitted).

Pascal Joset*, Régis Babey*, Andrin Wacker*, Esther A. Ingold, Esther T. Stoeckli, and Matthias Gesemann. MDGA2 is required for the growth of commissual axons along the longitudinal axis (in preparation).

Andrin Wacker and Esther T. Stoeckli. Wnt signaling, axon guidance and commissural axons (in preparation).

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